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ON PHOSPHATE EXCHANGE IN THE CENTRAL NERVOUS SYSTEM WITH SPECIAL REFERENCE TO METABOLIC ACTIVITY IN BARRIERS

By

LENNART HERLIN

STOCKHOLM 1956

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Ivar Hæggströms Boktryckeri AB

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Abbreviations

CNS = Central Nervous System

CSF = Cerebrospinal Fluid

BBB = Blood-Brain Barrier

BLB = Blood-Cerebrospinal-Fluid Barrier

LBB = Cerebrospinal-Fluid-Brain Barrier.

Preface

This investigation was carried out in the Department of Biophysics at the Wenner-Gren Institute for Experimental Biology, Stockholm, and in the Department of Pharmacology, Kungl. Veterinärhögskolan, Stockholm.

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LENNART HERLIN

CHAPTER 1

Introduction

Our knowledge of the metabolism of the brain is still very incomplete. Until recently the metabolism of the brain was most often considered to be small. This opinion has been difficult to disprove because of the lack of quantitative methods for studying metabolic and circulatory processes in the central nervous system (CNS) under physiological conditions. The era of isotopes in biological research (HEVESY) has provided new possibilities for studying detailed metabolic processes and turnover rates, which are also applicable to the central nervous system. Important contributions to our knowledge of brain circulation have lately been made by KETY (1948) and by GIBBS et al. (1947). These authors' procedures make it possible to calculate quantitatively the total cerebral blood flow in experimental animals and also in man. Local circulatory functions within the CNS, however, cannot be studied by these methods. The oxygen consumption and oxygen utilization can be calculated and thus it has been shown that the metabolism of the brain is large and comparable with that of other highly nourished organs such as the liver and the kidneys. Considering the very rich blood supply to the central nervous system, this is not surprising and can be expected a priori (WALTER 1933, FRIEDEMANN 1942, HIMWICH 1951).

Barriers within the central nervous system

Since EHRLICH's (1885) observation that, on subcutaneous injection, aniline dyes, especially acid dyes, hardly stained the CNS, the barrier problems of the central nervous system have attracted intense interest and numerous investigations have been

carried out to find the reasons why substances of very different kinds, introduced into or formed within an organism, cannot freely penetrate into the central nervous system. These phenomena have undoubtedly contributed to the opinion of many investigators that the metabolism of the central nervous system might be small. The existence of barriers in the central nervous system, however, does not permit such a conclusion (LINDBERG & ERNSTER 1950) since certain substances, after having penetrated into the central nervous system through the barriers, may remain there and participate in rapid metabolic processes which liberate considerable energy. Important components in metabolic systems, for example glucose, easily penetrate to the central nervous system.

The term "barrier" includes very complicated and different phenomena and earlier authors have described barrier processes of very different kinds. These authors have often used a terminology of their own and this has resulted in some confusion in addition to the confusion already existing concerning the phenomena themselves. Lina Stern and her school, for instance, have always used the term "Barrière hemato-encephalique" irrespective of fundamental differences such as delayed passage between the choroid plexus and the cerebrospinal fluid or delayed passage between the blood in the brain capillaries and the brain itself. This may have been due to the fact that the authors considered the histological conditions for the passage of a substance into the central nervous system to be similar, irrespective of the site of the process and the macroscopic conditions. In an analogous way, v. MONAKOW & MOURGUE (1928) make use of the term "Barrière ectomésodermique". At the time in question, it was generally considered that a substance penetrating into the central nervous system always had to pass a layer of cerebrospinal fluid. Each blood vessel which enters the brain from the surface is surrounded by a perivascular membrane forming funnel-shaped estuaries from the surface of the brain by the leptomeninges. The structure and function of these perivascular membranes have been the subject of intense interest on the part of many research workers. (PESTALOZZI 1849, VIRCHOW 1851, ROBIN 1859, HIS 1865, KEY & RETZIUS 1875, LEWANDOWSKY 1900, HELD 1909,

Mott 1910, Goldmann 1913, Weed 1914, Schaltenbrand & Bailey 1928, Flexner 1933, Hassin 1948 and many others).

There are many divergent opinions among the authors in this field and important structural and functional problems still remain unsolved. For instance, it is not quite clear whether there really exist perivascular spaces in open connection with the subarachnoidal space of the convexity of the brain, at any rate it is not clear how deeply into the brain such spaces can be followed from the surface. Recent ultramicroscopic investigations by Dempsey & Wislocki (1955) suggest that real perivascular spaces do not exist in the interior of the brain.

The composition of the extracellular fluid in the CNS is not known, nor is it known how the extracellular fluid circulates within the brain and in connection with the cerebrospinal fluid system. In any case, it is probable that the amount of such circulation is small.

Investigations concerning the barriers of the central nervous system have, as mentioned above, been numerous. It is therefore practically impossible to give a complete review of this subject. This is due not only to the great number of investigations in this field but also to the fact that the different methods of investigation contain sources of error which restrict the possibilities for drawing conclusions and comparing results.

The purpose of this review is to give a general idea of the problems involved in a vast physiological field and the literature referred to is fragmentary. For further reference the works of Flexner (1933), Friedemann (1942), Broman (1949) and Bakay (1956) are recommended. In this connection it may be worth mentioning that investigations using radioactive isotopes have the marked advantage that these isotopes can be determined with great accuracy in such minute amounts that they do not disturb the physiological conditions. This is of special importance when studying metabolic processes but also, of course, when studying the passage of electrolytes with no reference to metabolites.

Nevertheless, it must be emphasized that there still remain many technical difficulties in this field even when using isotopes. For instance, a piece of brain tissue taken as a sample in

experimental work will change its chemical composition within a few seconds. It is necessary to use methods which enable a very rapid fixation of the samples taken for investigation. In larger animals, such as rabbits, cats etc., this condition will be best satisfied by operating on living animals. In smaller animals, such as mice and rats, killing of the animal by rapid deep-freezing will often be a suitable method.

It is natural that the existence of barriers in the central nervous system, as well as in other parts of an organism, has led to systematic investigations to find out the nature of the barriers. As mentioned above, these investigations are theoretically and experimentally difficult and it is therefore practical to include in the term "barrier" all the phenomena which prevent, reduce, delay or even actually facilitate the penetration of a substance into the central nervous system. The penetration might occur by dialysis, ultrafiltration, osmosis, the Donnan equilibrium, electrical charges, lipoid solubility, special tissue affinity or metabolic activity. A more limited definition of this term would imply a more exact knowledge of the barrier mechanisms than one really possesses. If one had such exact knowledge, it would be unnecessary to use the term "barrier". It would be preferable merely to describe what happens. In reality, the barrier mechanisms may be different for each substance. This does not prevent certain phenomena from playing a dominant role in certain cases.

The barrier problems are intimately related to problems of active transport through cell membranes. Our knowledge of active transport systems is likewise incomplete. Under such circumstances it seems justified to include in the term "active transport" any translocation process in which the role of the cells appears to be more complex than that of merely offering a selective resistance to diffusion (LeFevre 1955).

Locally, the term "barrier" includes a functional synergism between many elements of tissue such as the choroid plexus, the arachnoidal villi, the ependyma, the leptomeninges, the membrana limitans gliae, the intrinsic cerebral blood-vessels, the membranes of the perivascular spaces and the membranes of the nerve cells.

Barriers and dyes

Among the numerous investigations concerning the barriers of the central nervous system, GOLDMANN'S experiments (1913) with dyes on living animals are acknowledged to be classical. On intravenous injection of large amounts of trypan blue (30—50 ml 1 % solution) in rabbits, all of the organs of the animals except the central nervous system were stained. There were no symptoms of poisoning. On the other hand, when small amounts of the dye were injected in the subarachnoidal space (0.5 ml, 0.5 % trypan blue), the animals developed convulsions and died within a short time and in these cases the central nervous system was stained. Similar conditions were found with dogs but dogs were not so sensitive to the toxic effect. The choroid plexus was moderately stained after intravenous administration of the dye and GOLDMANN considered the choroid plexus to be site of the barrier. SPATZ (1932), on the other hand, considers the choroid plexus in this connection rather as a "locus minoris resistentiae". Spatz agrees with RISER (1929) that the barrier in the GOLDMANN experiment is confined to the intima of the brain capillaries and he calls attention to the fact that the endothelium does not stain at all. This view has been confirmed by BROMAN (1940) and BROMAN & LINDBERG—BROMAN (1945). The results with dyes do not, of course, exclude the possibility that the barrier for other substances may be localized elsewhere or that several kinds of cell-layers may participate.

The experience gained through a great number of investigations soon necessitated a more differentiated concept of the barriers and various investigators began to distinguish between the blood-cerebrospinal-fluid barrier (BLB) and the blood-brain barrier (BBB). The choroid plexus is considered to be the main site of the BLB while the BBB is confined to the brain's intrinsic blood vessels.

The question of the site of the CSF-production is not yet solved in detail. Single observations of various kinds are not decisive. A great many experiences from different fields such as histology, pharmacology, pathology, embryology, direct observation of the choroid plexus during operations, studies in artificial hydro-

cephalus etc., together indicate that the main site of the CSF-production is probably the choroid plexus. This does not of course exclude other sources for the CSF-production, such as blood vessels in the leptomeninges, the ependyma and so forth. This is accepted by most authors on this subject and is confirmed by experiments of many kinds. So far, however, quantitative data in this field are but meagre. Investigations undertaken by SWEET & LOCKSLEY (1953) with the aid of radioactive isotopes confirm the opinion stated above and give good hope of providing more detailed knowledge in the future.

BERING (1955) has recently made isotopic investigations on the role of the choroid plexus in the exchange of substances between the blood and the CSF. Because of the results obtained, BERING has cast doubt on the importance of the choroid plexus in this respect.

Trypan blue is an acid dye and it was soon clear that acid dyes in general either do not penetrate the BBB at all or, if finely dispersed, only penetrate it to a small extent. Also, they can only penetrate the BLB to a small extent when present in high concentrations in the blood. On the other hand, basic dyes, *e.g.* methylene blue, easily penetrate the BBB but do not stain the CSF. (FLEXNER 1933, SPATZ 1933, BROMAN 1941, FRIEDEMANN 1942). This clear difference between the penetration of acid and basic dyes in the BBB indicates that the electrical charges may play an important part. This has been confirmed in several investigations, *inter alia* by FRIEDEMANN & ELKELES (1934) and FRIEDEMANN (1937), together with clinically important results obtained concerning neurotropic viruses. A positive electrical charge, or no charge at all, facilitates the permeability to the CNS from the blood while a negative charge prevents the penetration.

The electrical charges alone do not regulate the passage in the BBB. For instance, both cations and anions are more or less prevented from penetrating the BBB in a way which cannot be explained by consideration of the behaviour of the charges in the electrical fields. (WALLACE & BRODIE 1940, BRODIE & WALLACE 1940, NOONAN, FENN & HAEGE 1940, HAHN & HEVESY 1941, KROGH 1946).

The lipoid solubility

Lipoid solubility is another factor which seems to facilitate penetration through the BBB to the CNS (KROGH 1946). This is well-known from the rapidity with which narcosis can be obtained by the use of lipoid-soluble anaesthetic drugs. The ability of basic dyes to pass the BBB seems to be connected, at least partly, with their lipoid solubility.

Distribution of inorganic ions in the BLB

The distribution of electrolytes between the blood and the CSF has been the subject of many investigations, *e.g.* the experiments of WALTER (1925) with sodium bromide, showing a typical and rather constant ratio plasma/CSF, *viz.* about 3/1, indicating that the passage from the blood to the CSF is impeded. STARY, KRAL & WINTERNITZ (1929) showed that some ions, such as Na^+, Ca^{2+} and Cl^-, distribute between the blood and the CSF in agreement with the physical laws of dialysis or ultrafiltration while others, such as Mg^{2+}, K^+ and PO_4^{3-} show divergences from these physical laws. According to our present knowledge this is not surprising since Mg^{2+}, K^+ and PO_4^{3-} are known to form organic complexes whereas Na^+ and Cl^- are less subject to complex formation. K^+ and PO_4^{3-} have a restricted passage through the barrier whereas the concentration of Mg^{2+} is higher in the CSF than in the blood. Other contributions in this field have been made by, among others, KRAL, STARY & WINTERNITZ (1929), FLEXNER (1934), MCCANCE & WATCHORN (1934), KAFKA (1938), WALLACE & BRODIE (1940), and the authors conclude that there must be a selective, active transport mechanism in the barrier. However, there are also authors who regard the existence of active transport mechanisms in this connection as not completely proved (PINCUS & KRAMER 1923, WITTGENSTEIN & KREBS 1926, WALTER 1931, MERRIT & FREMONT—SMITH 1937). Many of the investigations mentioned above have the disadvantage that the electrolytes have been administered in doses which were so high that they disturbed the physiological conditions.

Isolated objections concerning the pentration of different substances in different ways to the CNS, *e.g.* that molecular binding to proteins in the blood does not permit free diffusion and that the transport away from the CSF is unknown and so forth, may indicate that some conclusions are erroneous. On the whole, however, the theory that there exist different forms of selective, active transport mechanisms in the BLB as well as in the BBB must be considered very plausible. The investigations of GREENBERG *et al.* (1943) on the BLB, in which radioactive isotopes were used in concentrations which had no material effect on physiological conditions, support this opinion.

The production of CSF

The choroid plexus is considered, for good reasons, to be the main site of the CSF production. It is built essentially as a secretory organ (*viz.*, a large number of capillaries close to epithelial cells which are arranged in crinkled structures of large surface area).

It is reasonable to presume that the choroid plexus strictly regulates the composition of the CSF and this implies that there are barrier mechanisms in the plexus between the blood and the CSF. By analogy to other secretory organs, the metabolism of the plexus can *a priori* be presumed to be high, and that this is also the case has been shown in cultures of the tissue by KREBS & ROSENHAGEN (1931). The metabolism of the choroid plexus is of a magnitude comparable with that of the brain itself and that of parenchymatous organs such as the liver and the kidneys. This means that the CSF is formed in cells capable of active secretory work and this gives strong support to the hypothesis that the formation of the CSF not only occurs according to physical laws but also includes active work of secretion. As has been emphasized by, *inter alia*, KROGH (1946), it is typical for such active processes that they consume energy in order to be maintained. The kind of cells that take part in the active work of the choroid plexus is not yet completely known.

The question of a barrier between the CSF and the CNS

There are divergences of opinion concerning the existence of a barrier between the CSF and the CNS. Many authors deny the existence of such a barrier (LEWANDOWSKY 1900, GOLDMANN 1913, SCHMID 1931, ROEDER 1948, BAKAY 1951) while others accept the idea of this barrier on general grounds. WALTER (1932) believes in this barrier but considers it to possess passive properties of a semipermeable nature in the limiting surfaces. SCHORRE (1942) expresses a similar point of view. JAHN (1940) argues in favour of this barrier on the basis of detailed and careful anatomic pathological investigations but the arguments advanced do not seem quite clear. Experiments with dyes in the CSF (GOLDMANN 1913, WEED 1914) prove little concerning the existence of a barrier. Weed in his investigations did not think of barrier problems in this connection. His intention was to study the problems of CSF-circulation.

During investigations on the BBB, experiments with dyes have shown, as mentioned above, that the intima endothelium of the blood vessels prevents acid dyes from penetrating to the CNS. Considering the complicated processes involved in this barrier from a wider point of view, it is reasonable to assume that different cell-layers may have different functions. In this connection (GÄRTNER 1927, SCHALTENBRAND & BAILEY 1928), it is the walls of the perivascular spaces which attract attention. We know that the brain swells after intravenous injections of hypotonic solutions and shrinks after the injection of hypertonic solutions (WEED 1923). SCHALTENBRAND & BAILEY (1928) showed in experiments on narcotized cats that the contrary conditions are found with the cells in the pial-glial membrane, suggesting a counterbalancing of too great differences in osmotic pressure. Tumour-cells often show a different histological appearance according to whether they proliferate inside or outside the perivascular membrane and SCHALTENBRAND & BAILEY felt that the reason for the different appearance might be the different conditions for nourishment. These authors also refer to the investigations of the Spanish histologists NAGEOTTE (1910), ACHÚCARRO (1913) and RAMÓN Y CAJAL (1913) showing glial cells

rich in mitochondria. According to Nageotte, the histological properties indicate that the perivascular membrane, especially its glial elements, may be of importance as a barrier for metabolites. However, his arguments might of course be equally valid for the convexity of the brain where the pial-glial membrane has no close relation to the bloodvessels, that is to say, the membrane may play an active part in a barrier function between the CSF and the CNS.

However, there are also surfaces separating the CSF from the brain in the ventricular system and, in this connection, the ependyma is of interest (JAHN 1940). In a monograph of 1928, v. MONAKOW & MOURGUE, referring to the histological investigations of GRYNFELTT & EUZIÈRE (1913), consider the probability of the ventricular ependyma having a secretory function.

Direct evidence for the existence of a barrier between the CSF and the CNS was given by HERLIN (1954) who demonstrated considerable differences in the specific activities of orthophosphate for the CSF and the brain in living rabbits about one hour after the injection of radioactive orthophosphate in the cisterna cerebello-medullaris. The barrier was also clearly illustrated in autoradiographs of rats' brains with the same isotope. From these experiments it is not, of course, possible to say anything about how multifarious this barrier may be.

Reasons for the author's experimental work

With regard to the complexity of problems referred to in this introduction, an investigation of the exchange of carrier-free radioactive orthophosphate in the CNS was performed for the following reasons:

a) Radioactive isotopes can be determined with great accuracy in experimental animals even after the administration of minute amounts insufficient to influence the physiological concentrations.

b) Previous investigations of the barriers in the CNS using radioactive isotopes are comparatively few.

c) It was considered probable that the barrier mechanisms

of the CNS include not merely passive physical processes but also processes of active transport.

d) Among processes of active transport presumed to play a part in the barriers of the CNS, metabolic processes must also be taken into account (USSING 1953).

e) Investigations on metabolic processes in the CNS with special regard to barriers are scarce.

f) Orthophosphate is a very important substance in connection with metabolic processes of different kinds. It was considered probable that experimental studies of barriers in the CNS with the aid of radiactive orthophosphate ought to reveal not only the site of barriers but also the occurrence of metabolic processes.

g) Metabolic processes of complicated nature and specific localization are likely to be sensitive to external and internal disturbances. Knowledge in such a field can be excepted not only to have a purely theoretical interest but also to become a fundamental basis for a more profound understanding of different pathological conditions and how they arise. Such increased knowledge may suggest possible protective and curative measures.

CHAPTER 2

BLB and BBB in the Rabbit Shown by Means of Determinations of Specific Activities of Orthophosphate

The existence of these barriers has been proved earlier in different kinds of animals with radioactive orthophosphate. Thus, GREENBERG *et al.* (1943) examined several different isotopes, among them orthophosphate, with regard to their passage from the blood to the CSF in dogs. Samples from only the blood and the CSF were taken, leaving possible transitory organs (*e.g.* the choroid plexus) and receiving organs (*e.g.* the brain) intact.

Several authors (COHN & GREENBERG 1938, HEVESY & HAHN 1939—41, JONES, CHAIKOFF & LAWRENCE 1940, FRIES & CHAIKOFF 1941, MANERY & BALE 1941, BORELL & ÖRSTRÖM 1945, LINDBERG & ERNSTER 1950, BAKAY 1951 and 1954, SAMUELS *et al.* 1951) have demonstrated the BBB using radioactive orthophosphate. All these authors killed their animals to get samples from the brain.

In this chapter, the present author reports results from samples taken directly from living rabbits.

Definitions and assumptions

The term "specific activity" refers to the radioactivity of one unit of weight of a radioactive substance. The specific activity can be obtained as a quotient of the radioactive part over the non-radioactive part of the substance in question and can be expressed in different ways. A usual way of expressing the specific activity is to state the number of impulses per minute

obtained from 1 mg of the substance referred to. The numerical values for the specific activities of the samples become less unwieldy if calculated on a unit of weight of 10^{-3} mg phosphorus (γP), and have been expressed as such in this experimental work. In order to compare the results obtained for the different animals in a series, it is justifiable and practical to express both the total radioactivities and the specific activities as percentages of one of the values, usually the highest value found (100 %). This method of expressing the radioactivities (relative radioactivities) is especially to be recommended when studying the variations of the radioactivities with time.

Supposing that a radioactive substance can pass freely from one tissue to another or from a fluid of the body to a tissue, a determination of the specific activities for the two tissues or for the fluid and the tissue will give results of similar magnitude, regardless of the physiological concentrations of the substance in a non-radioactive form on both sides. If the specific activity of a radioactive substance on the side of administration remains high in comparison with the other side for a longer time than is to be expected according to physical laws, this indicates a prevented or delayed passage to the other side. This argument holds true even if the radioactive compound administered forms new chemical compounds on one or both sides. A necessary assumption for the validity of this statement is that the radioactive compound administered represents a residual fraction on both sides and that this fraction can be isolated.

Experimental

Operative procedures

Five rabbits were used. The animals were injected intraperitoneally with a dose of radioactive orthophosphate varying within approximately 0.2—1 mC P^{32} in 0.2 ml physiological saline, according to PALM (1948). From an intraperitoneal depot, orthophosphate is rapidly absorbed into the blood chemically unchanged (PALM 1948) and the maximum content of P^{32} in the blood is reached within about 30 minutes.

Blood samples were taken from a vein in one ear during the first 30 minutes at intervals of 5 minutes, during the second 30 minutes at intervals of 10 minutes and during the second hour at intervals of 15 minutes. For each blood sample, 5—6 drops were collected in a centrifuge tube containing 1 ml of 15 % trichloracetic acid (TCA) kept ice cold to prevent hydrolysis of unstable organic phosphoric compounds.

Subsequently, each animal was operated upon under narcotal narcosis, administered intravenously in a dose of 50 mg per kg body-weight immediately before the operation. Narcotal is a quick-acting Swedish barbiturate preparation (*iso*-propyl-*β*-bromoallyl-N-methylmalonylcarbamide sodium). The convexity of the skull was removed, leaving the dural covering intact, and the membrana atlanto-occipitalis was exposed. After this, the various end samples were taken, the time interval between them being kept as small as possible. With trained hands, all samples can be taken within a couple of minutes and the results are judged and compared as if the end samples had been taken at the same time. The first end sample is a CSF-sample taken with a whole-glass syringe of 1 ml capacity after puncturing the membrana atlanto-occipitalis near the cranial vertex of the foramen magnum, accurately in the midline, to a depth of about 2 mm. It is advisable to use a fine needle with a short cut point. In order to avoid making the puncture too deep, the needles were threaded through a thin piece of cork leaving 2 mm at the end of the needle free. This greatly facilitates the taking of CSF-samples. If the CSF does not enter the syringe immediately the piston is retracted, it will usually do so if the syringe is rotated carefully, and a CSF-sample of a volume of 0.5—1 ml can be taken without much difficulty. The CSF does not undergo chemical changes of importance and the CSF-sample can be left in the syringe for a while.

When the CSF-sample had been secured, the dural covering of the skull convexity was opened, and a small piece of brain tissue weighing about 20 mg was taken with a thin, sharp spatula from the convexity of one of the parietal lobes. The sample of brain tissue thus taken was immediately fixed by grinding it in a small porcelain mortar together with 0.4 ml 10 N H_2SO_4. During

these experiments similar samples were also taken at random from other parts of the brain. They yielded no significant differences during the subsequent investigations.

When the brain sample had been taken, the brain tissue covering the lateral ventricles was divided and the choroid plexus on one or both sides was removed with a pair of small pincers. This sample was immediately fixed in the same manner as described for the brain sample.

Finally a blood-sample was taken, this being the last one in the series.

The operations have been performed using the neurosurgical technique and equipment, and the animals were kept in good condition during the whole of the procedure.

Determinations of specific activities

The specific activities for the orthophosphate fraction of all the samples were determined according to ERNSTER, ZETTERSTRÖM & LINDBERG (1950), including chemical analysis according to MARTIN & DOTY (1949) and determination of radioactivities in a Geiger-Müller counter.

Results

The specific activities for whole-blood follow a time curve of the same type as that found by HEVESY (1939) and PALM (1948) for plasma. This is not surprising considering that the relatively small part of the orthophosphate which enters the corpuscles of the blood is almost completely bound as organic compounds (HEVESY 1940).

The results concerning the barriers are essentially the same in all five cases. There is a definite hindrance to the free passage of radioactive orthophosphate from the blood to the choroid plexus as well as to the CSF and the brain.

A typical experiment is shown in fig. 1 (Rabbit No. 1).

The results from all the cases are given in Table 1. Of the blood samples, it is only the end sample which is of interest for

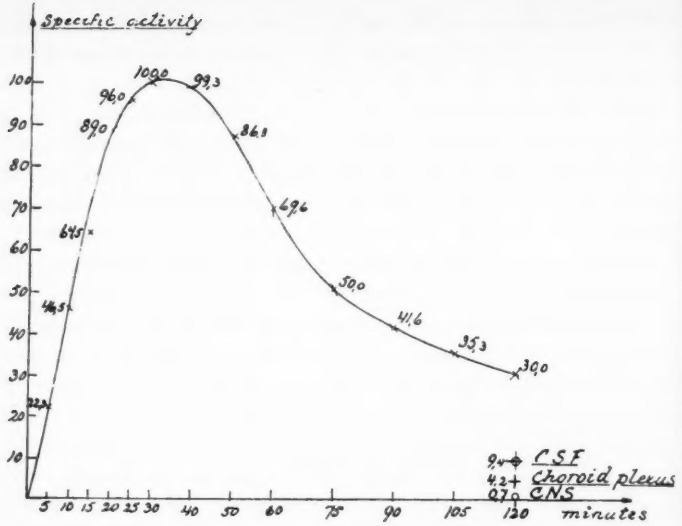


Fig. 1. Example of determinations of specific activities following intraperitoneal injection of radioactive orthophosphate in a rabbit (Rabbit No. 1 in Table 1). The curve indicates specific activities in the blood at various time intervals. All the specific activities have been expressed as relative specific activities.

judging the results. The values of the other blood samples have therefore been excluded.

Discussion and conclusions

As regards the choroid plexus, the specific activities for the four experiments lasting two hours averaged 15.6 % of the specific activity in the blood at the same time, 20.4 % being the highest value found (Rabbit No. 4) and 11.1 % the lowest (Rabbit No. 2). The corresponding value for the choroid plexus in the experiment lasting one hour was 17 %.

If the radioactive orthophosphate of the blood could pass over to the plexus freely, one would find a specific activity in the

Table 1

Rabbit		Time after injection of P^{32} in minutes	Blood orthophosph. fraction			Choroid Plexus orthophosph. fraction			CST orthophosph. fraction			CNS orthophosph. fraction		
No	Weight kg.		Counts per min.	γP	Specific activity	Counts per min.	γP	Specific activity	Counts per min.	γP	Specific activity	Counts per min.	γP	Specific activity
1	2.0	120	415	4.83	86.0	81	6.72	12.0	126	4.61	27.0	33	16.95	2.0
2	2.0	120	927	8.82	105.0	52	4.45	11.7	37	3.47	10.4	24	8.14	2.9
3	2.0	120	523	21.10	24.8	28	5.60	5.0	29	3.47	8.4	5.2	4.90	1.1
4	1.3	120	420	4.18	100.4	95	4.65	20.5	54	1.47	36.7	10.4	3.26	3.2
5	2.4	60	129	3.78	34.2	16.2	2.77	5.8	21	3.52	6.0	9.4	4.44	2.1

choroid plexus of a magnitude similar to that found in the blood at the same time. The values found show that there is already a hindrance to the passage of P^{32} in the blood vessels of the choroid plexus and thus it is clear that the blood-vessels of the choroid plexus possess a barrier function. This fact has not been directly proved before and previous authors, with the support of dye experiments, have most often been of the opinion that the barrier process in the choroid plexus is localized in the epithelium cells of the plexus.

The fact that the blood vessels of the choroid plexus have a barrier function does not, of course, exclude the possibility that the epithelium cells of the plexus may also play a part in the complicated mechanism composing the entire BLB. Indeed, this is very probable in view of the dye experiments where the whole of the plexus becomes coloured from the blood with little entrance of colour to the CSF (GOLDMANN 1913, SPATZ 1933, BROMAN 1949). There are signs indicating that the epithelium cells also play a part in the penetration of orthophosphate (see Chapters 3, 4 and 8).

In the CSF, the specific activities show somewhat higher values than those found in the choroid plexus at the same time, except in case No. 2, where a slightly lower value was found. The real specific activities of the choroid plexus ought to be somewhat lower than those found in the experiments with regard to the normal blood content of the plexus. This may imply that the epithelium of the plexus facilitates the entrance of the orthophosphate to the CSF or that the CSF may have other sources than the choroid plexus for its orthophosphate content. On the whole, however, the differences in specific activities obtained in these experiments are too small to permit any significant conclusions with regard to these arguments, a circumstance that can hardly be changed by examining the normal blood content of the choroid plexus.

The very low specific activities found in all cases for the brain tissue agree with the results found by earlier investigators and demonstrate the presence of a considerable barrier towards orthophosphate in the BBB.

CHAPTER 3

Direct Evidence for the Existence of the LBB in the Rabbit by Means of Determinations of Specific Activities of Orthophosphate

The very strong hindrance to the penetration of orthophosphate into the brain by the BBB supports the opinion that the brain may get an essential part of its orthophosphate requirements via the BLB (SACKS & CULBRETH 1951, BAKAY 1956). The BLB is certainly also an important barrier towards orthophosphate but is not so strong as the BBB.

Several authors, however, have shown that the BBB is not of uniform strength in all parts of the brain. Trypan blue, for instance, always enters the BBB in certain welldefined areas of the brain, *viz.* the hypophysis, the infundibulum, the tuber cinereum, the area postrema, the paraphysis, the corpus pineale and the recessus preopticus. These areas transmit many substances more freely than other parts of the brain (SCHULEMANN 1912, RACHMANOW 1913, PUTNAM 1922, MANDELSTAMM & KRYLOW 1927, SPATZ 1933, KING 1939, GRUNER *et al.* 1951, BAKAY 1951, 1952). As shown by BORELL & ÖRSTRÖM (1945), this is also valid for radioactive orthophosphate as far as the corpus pineale, the hypophysis, the tuber cinereum, the substantia perforata and the choroid plexus are concerned. The present author was able to confirm these findings concerning the hypophysis and the choroid plexus of rats, since a definitely stronger activity in these parts as compared with other parts of the brain can be directly seen in autoradiographs following intraperitoneal injection of labelled orthophosphate. The activity of the choroid plexus on the autoradiographs seems to be located in the blood-

vessels of the plexus. This fact does not contradict the findings of BORELL & ÖRSTRÖM (see Chapter 4 page 39—41).

In addition to the choroid plexus, part of the BLB, there are thus also small parts of the brain which belong to the BBB, where the penetration of orthophosphate is facilitated as compared with the rest of the brain. It is probable that the brain gets part of its orthophosphate requirements via these parts of the brain. This may explain the fact that the P^{32} activity in the brain continues to increase slightly even when the activity of the blood has approached zero (JONES *et al.* 1940, SAMUELS *et al.* 1951, BAKAY 1952, ABOOD *et al.* 1952). In this connection, however, it is difficult to exclude the blood as a source for the increasing activity since the maximum activity of the whole brain never reaches more than a very low value. Special investigations are necessary to disentangle these complicated problems.

Of great interest in this connection are the works of BAKAY & LINDBERG (1949) and LINDBERG & ERNSTER (1950) showing a surprisingly rapid passage of P^{32} from the CSF to the CNS resulting in an extensive and rapid formation of complexes with organic compounds in the CNS (40 % of the P^{32} in the CNS was found to be bound two minutes after the CSF injection). In a discussion with the author on these problems, Lindberg remarked that the rapid passage of P^{32} from the CSF to the CNS was in his opinion an active process.

BAKAY (1951), in an autoradiographic study of barriers with labelled orthophosphate in rabbits, did not find a barrier between the CSF and the CNS. To secure the LBB, however, samples ought to be taken on living animals, or else the specimen must be kept constantly cold. The present author has noticed that sectioning of the brain for autoradiography in a room not sufficiently cold will destroy the signs of the LBB, probably by causing the P^{32} to flow on the sectioned surface. Sectioning at a temperature above about —8° C must therefore be avoided (See Chapter 4).

The results reviewed and discussed in this and the preceding chapter made it of interest to study the passage of radioactive orthophosphate from the CSF to the CNS in living animals by

means of determinations of the specific activities of the orthophosphate fractions of the CSF, the choroid plexus and the CNS after the injection of the phosphate into the cisterna magna.

Experimental

Four rabbits have been used for these experiments. The animals were narcotized with narcotal using a dose of 50 mg per kg bodyweight at the beginning of the experiments. The membrana atlanto-occipitalis was exposed. The animals were injected in the cisterna magna with labelled orthophosphate contained in a volume of physiological saline of 0.1 ml, the dose of P^{32} varying within approximately 0.05—0.1 mC. The technique of puncturing the cisterna magna was the same as in the preceding chapter (see page 22). For the injections, tuberculine syringes of 1 ml with glass pistons and graduated to 0.01 ml were used. Thin injection needles (No. 20) with short cut points were used. It is not recommended to evacuate a corresponding volume of CSF before the injection since experience shows that it is difficult to get CSF into the syringe by retracting the piston after a second puncture and since this would increase the risk of getting a false injection outside the arachnoidal membrane. The radioactive orthophosphate injected diffuses rapidly in the CSF. A leakage backwards through the injection hole can be stopped by gently pressing a piece of muscle against it. The leakage stops very soon and does not materially affect the results.

In order to obtain samples from the living animals, operations were performed in the same manner as in the preceding chapter. The samples were taken at varying times after the injection, *viz.* 40 minutes in one case (No. 1), 80 minutes in one case (No. 2) and 60 minutes in cases No. 3 and No. 4.

Results

The results are given in Table 2.

Table 2

Rabbit		Time after injection of P^{32} in minutes	CST orthophosph. fraction			CNS orthophosph. fraction			Choroid Plexus orthophosph. fraction		
No.	Weight kg.		Counts per min.	γP	Specific activity	Counts per min.	γP	Specific activity	Counts per min.	γP	Specific activity
1	2.6	40	4989	7.5	670	42	13.5	3.1	2	13.8	0.1
2	2.5	80	21328	—	~4000	3	13.3	0.3	4	—	Low
3	2.7	60	23117	6.0	3900	377	15.4	24.5	43	8.2	5.2
4	1.7	60	4596	5.6	820	16	12.3	1.3	11	6.1	1.8

Discussion and conclusions

From the table it is immediately evident that both the total activities and the specific activities for the choroid plexus, as well as for the brain, are very low. An exact calculation of the γP of the CSF and the choroid plexus in rabbit No. 2 was not possible because of a technical mishap. This is of no essential importance since the total radioactivities of samples of both the choroid plexus and the brain are very low. This also ensures that the samples have very low specific activities. The relatively high total activity in the CSF gives a specific activity for the CSF of about 4000. The main point is that the specific activities for both the choroid plexus and the CNS are very low compared with the specific activity of the CSF.

From the results, the conclusion can be drawn that the orthophosphate meets a barrier on its penetration from the CSF into both the choroid plexus and the CNS.

As regards the choroid plexus, it is obvious that the epithelium of the plexus forms a barrier for the penetration of orthophosphate from the CSF to the choroid plexus. In the BLB, one must consider that both the blood vessels and the epithelium of the choroid plexus participate in a barrier mechanism for orthophosphate. This is *a priori* probable also for many other substances. The fact that the epithelium of the plexus restricts the orthophosphate penetration in the direction CSF → choroid plexus does not permit the conclusion that this is also the case for the opposite direction (Chapter 2 page 26). Bipolar or irreciprocal properties of biological membranes are well-known in other connections. Thus, for instance, frogskin and the cornea of many animals usually permit the inward passage of acid dyes, whereas the passage of basic dyes is usually in the opposite direction (WERTHEIMER 1923, FRIEDEMANN 1937, BROMAN 1941).

FLEXNER (1933), in a discussion of the results of several investigators, considers the possibility of the choroid plexus having irreciprocal properties. FLEXNER, however, points out that the investigations cited do not admit conclusions for physiological conditions.

As regards orthophosphate in physiological concentrations, the

present author's experiments show that the blood vessels of the choroid plexus contain a barrier for the penetration in the direction blood → CSF and that the epithelium of the plexus constitutes a hindrance in the direction CSF → choroid plexus. Facts regarding the penetration of orthophosphate in the other possible directions within the choroid plexus are not known (compare the results from Chapter 2).

With regard to the barrier towards orthophosphate between the CSF and the CNS, it seemed probable that samples from the outermost layer of the brain should give higher activity values, but a technique for getting such samples would be extremely difficult. It was considered more satisfactory to elucidate the problem using a new approach (See next chapter).

In any case, the existence of the LBB towards orthophosphate can now be considered proved. In view of the fundamental role of orthopsphate in intermediary metabolism and the fact that 40 % of the P^{32} was found to have formed organic complexes in samples of the brain taken 2 minutes after injection of labelled orthophosphate in the CSF (LINDBERG & ERNSTER 1950), it is probable that active metabolic processes are involved in the LBB. It also seems probable that metabolites produced by such active metabolic processes in the bounding tissue-layers of the brain will be gradually transported into the interior of the brain. Autoradiographic studies show that such is the case (see Chapter 4, page 46). It seems likely that certain cells in the interior of the brain may have a strong metabolic affinity for inorganic phosphate contained in the extracellular fluid. The glial cells in the bounding surfaces of the brain, however, have a histological structure of their own. The conditions under which such cells can absorb inorganic phosphate in metabolic processes are not identical with those for cells in the interior of the brain. It does not either seem likely that the metabolic processes in nervous cells of different kinds are identical. The delayed entrance of P^{32} from the bounding surfaces of the CNS to the interior of the CNS probably indicates that the penetration of P^{32} involves active transport mechanisms of different kinds.

CHAPTER 4

Autoradiographic Studies of the BBB, the BLB and the LBB in Rats Using Radioactive Orthophosphate

In Chapters 2 and 3, the BBB, the BLB and the LBB for orthophosphate were demonstrated by means of experiments on living rabbits.

Biological experience indicates that phenomena of this kind are essentially of much the same nature for many different kinds of animals. In view of this, it was desirable to illustrate the results obtained in Chapters 2 and 3 by another approach and on another species of animal. If the results thus obtained are equivalent to those reported in Chapters 2 and 3, then they would support the conclusions drawn in those chapters. Such an equivalence would at the same time support the view, founded on biological experience, that one is dealing here with phenomena which are essentially similar in different animals.

Autoradiography was chosen as the experimental method because it has the advantage of giving a direct visual picture of the barriers mentioned in Chapters 2 and 3. When relatively small animals are used, errors due to the post-mortem hydrolysis and diffusion of the administered P^{32} can be prevented by killing the animal by rapid freezing. For this purpose, rats were chosen as experimental animals. The use of rats has the further advantage of making possible the preparation of complete sections of the whole of the brain for autoradiography. In order to prevent errors due to diffusion, the sections which had been cut were kept cold during the whole autoradiographic procedure. In order to avoid the hydrolysis and diffusion errors mentioned above, no other fixation method except rapid freezing was used. It was

considered that these precautionary measures would make the results obtained reliable. On the other hand, the experiments have not been intended to be quantitative, nor do the results make it possible to localize the activity seen in the autoradiographs with cellular exactness.

Experimental

White laboratory rats of both sexes weighing about 150 grams have been used. After each experiment, the animal was killed by freezing in a mixture of acetone and dry ice at a temperature of about —75° C. This freezing mixture flows easily and rapidly penetrates the fur.

Preparation of the rats

In order to demonstrate the BBB and the BLB, a convenient dose of radioactive orthophosphate, about within 0.05—0.25 mC dissolved in 0.1 ml physiological saline, was injected intraperitoneally into the animals without narcosis. From the intraperitoneal depot, the activity will be absorbed by the blood in the same manner as described for the rabbits above (page 23). The animals (three in number) were killed after 30, 35 and 45 minutes.

In order to demonstrate the LBB, labelled orthophosphate was injected directly into the cisterna magna about 10 minutes after the injection of 0.4—0.5 cc narcotal intraperitoneally for narcosis. The dose of P^{32} injected was about 2—5 μC dissolved in a volume of 0.01—0.02 ml physiological saline. In all, nine animals were prepared in this series, the time of survival after the injection varying from 1 minute up to 60 minutes. In addition, several animals were prepared in the beginning in order to determine the adequate doses of P^{32} and the most suitable film for autoradiography.

Tuberculine syringes of 1 ml capacity, with glass pistons and graduated to 0.01 ml, were used. The needles, thin (No. 20) and with short cut points, were used only once.

Technique of injecting into the cisterna magna

For the cisterna magna injection, the following technique was used:

The rat, anaesthetized, is strapped on a stand for puncture of the cisterna magna as described by JEFFERS & GRIFFITH (1942). The punctures of the cisterna magna were performed according to a technique worked out by the present author. The procedure is as follows: A small piece of skin is removed between the external occipital protuberance and the spinal process of the second cervical vertebra—these are both anatomical structures which can be easily detected by touch. The distance between them is as much as about 1 cm. The puncture should be performed in the midline immediately cranial to the spinal process of the second cervical vertebra. A superficial artery in the midline is always present and can easily be avoided. The needle is introduced almost horizontally until its point reaches the occipital bone in the midline and cranial to the foramen magnum. Thereafter, the position of the point of the needle is gradually altered, successively approaching the cranial vertex of the foramen magnum by repeatedly prodding the occipital bone. Immediately before the edge of the foramen magnum is reached, one can feel very clearly a slight depression of the bone and, on the next thrust, the needly will slip under the edge of the foramen magnum to a depth of about $^1/_2$ mm into the cisterna magna, no resistance being noticed at this point. The selected dose of radioactive phosphate dissolved in a volume of 0.01—0.02 ml physiological saline is then injected.

Technique for preparation and sectioning of the brains

Death by freezing is rapid. The body is taken out from the freezing mixture after about 15 minutes. The head of the rat is placed in a freeze-room kept at a constant temperature of —11° C. The superficial soft tissues are removed from the skull. Then the base of the skull is frozen to a microtome block by means of a piece of cotton wool dipped in ice water. Thereafter the bony convexity of the skull is removed.

The brains were sectioned in a Leitz sledge-microtome, type 1300, the thickness of the sections usually being 15 or 20 μ according to the scale of the microtome. This procedure is technically difficult, the technique employed by ULLBERG (1954) for the complete bodies of mice being used. As recommended by PALMGREN (1954), the section is stuck to a strip of transparent tape.

The sections are left to dry for 24 hours. The present author believes that the sections dry quickly, because it can be noticed that the sections become more fragile not more than one minute after cutting. It is also possible to see with the naked eye that the choroid plexus begins to shrink after a similarly short time.

Several modifications of the sectioning work were tried in order to find out the most convenient method. The best sectioning plane turned out to be a horizontal plane which included the olfactory lobes, the hemispheres with the lateral ventricles, the brain stem and the cerebellum. Other planes showed no essential differences but did not give such a good general picture of the contents of the brain. Skulls have also been cut with the bone intact. The sections in this case were not, of course, as regular as those obtained when the bony coverings had been removed. It was found that the results obtained with such sections exhibited no important differences. When autoradiographs of basic parts of the brain were desired, the bones of the skull had to be left intact. In such cases, the skull was mounted on the convexity, with the base turned upwards so that it could be cut with the microtome knife.

Technique for producing the autoradiographs

The apposition technique of autoradiography was considered to be suitable. Kodak X-ray film Kodirex Code 1 was used. This film has a rather high speed and consequently a comparatively coarse grain. The resolution is not sufficient to permit the study of single cells. However, radioactive orthophosphate is a powerful emitter of β-rays which concentrates in these experiments to very restricted areas representing the barriers. For this reason little will be gained by using a film of finer grain. Moreover,

fine grain films have the disadvantage of requiring a longer exposure time.

Exposure. The strips of tape bearing the sections were stuck to suitable pieces of film (35 × 75 mm) in the freeze-room which was also used as a darkroom. Data concerning the sections had previously been marked on the adhesive side of the tape with radioactive India ink, *i.e.* ink containing a suitable amount of P^{32} (ULLBERG 1954). All the work in connection with the exposure was performed in the indirect light from a Kodak safelight (Wratten No. 6). Up to ten such units consisting of section + film were piled on one another, separated by methacrylate slides, pieces of flannelette and cardboard and then put into iron presses of the same kind as those used by ODEBLAD (1952). During the exposure, the iron presses were stored in light-tight metal boxes. The exposure time was usually 4—6 days.

Development. The presses were unpacked in a darkroom kept at 20° C. The pieces of film were separated from the sections by removing the strips of tape. It may be difficult to avoid an "unspecific" scattered blackening on the film where the tape had been stuck, probably owing to electrical discharges during the separation. This scattered blackening, however, is situated peripheral to the sections and does not influence the autoradiographic pictures. Development of the films was performed using the developer Kodak 19 B for 5 minutes. Johnson 326 wetting agent (1 ml) was added to each litre of developer. Development was interrupted using an acid stopping bath consisting of 2 per cent acetic acid in tap water.

Fixing and washing. Fixing was carried out using Kodak fixing bath F 5 for 30 minutes. After fixing, the films were washed in running tap water for about two hours. The films were dried in air. Most of the sections were stained and mounted without removing the sections from the tape. The usual stain, haemalun, was used. The stain was rinsed off with tap water and the strips of tape were allowed to absorb water. The sections were then mounted on a thin layer of albumin-glycerine painted on an object-glass and the wet tape fastened with the section side against the albumin-glycerine on the glass. The tape served as a cover-glass. Excess water was removed with blotting paper.

The tape was covered with several layers of absorbent paper and a pile of such sections were put in press for about 24 hours. A few sections in the later course of this work were removed from the tape and mounted in Canada balsam according to PALMGREN (1954). This procedure is preferable at high magnification.

Results

A. *BBB*

The autoradiographs obtained after intraperitoneal injection of labelled orthophosphate into rats illustrate the BBB and the BLB very clearly (Fig. 2). Since the autoradiographs do not permit accurate measurements or comparisons, it has not been possible to describe the results in this chapter quantitatively. In all large blood-vessels, it is seen that a strong activity has affected the film due to the blood content whereas the brain itself only shows a very weak activity. It is not possible to judge how much of the weak activity in the brain has left the brain-capillaries or how much of it is due to the normal content of capillary blood in the brain. Parts which normally are rich in blood capillaries, *viz.* the cortex, possess a radioactivity which, although not very large, is greater than that of other parts of the brain. These results seem to be quite in agreement with those of previous authors obtained by other approaches to the problem (for references see Chapter 2, page 20).

In Chapter 3, page 27, it was pointed out that several authors have shown that the BBB is not of uniform strength in all parts of the brain. Certain well-defined parts pass many substances, including orthophosphate (BORELL & ÖRSTRÖM 1945), better than the rest of the brain. In the present work, no special emphasis has been placed on this problem. The results of BORELL & ÖRSTRÖM, however, have been confirmed as regards the hypophysis. A stronger activity in the hypophysis can be clearly seen in the autoradiographs, as compared with other parts of the brain (Fig. 3). The non-uniformity of the BBB is of great interest with regard to the hypothetical transport pathways of metabolites and other substances to the interior of the brain (see page 28).

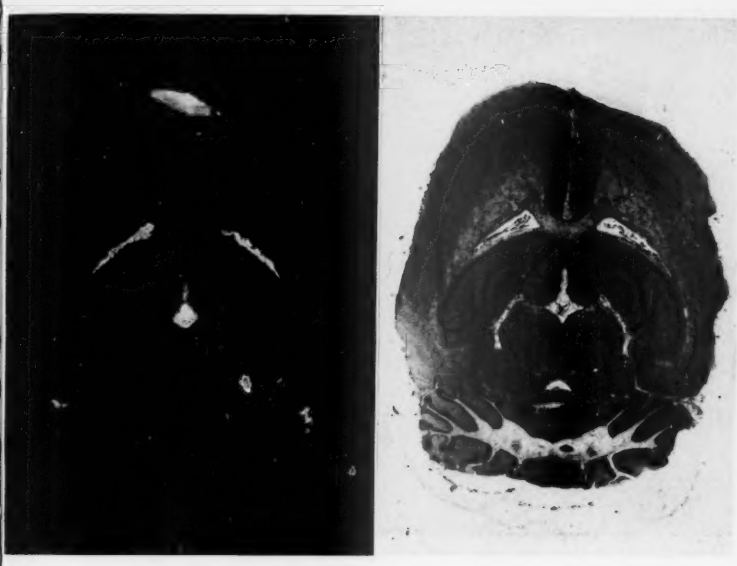


Fig. 2. BBB and BLB illustrated by autoradiography of rat brain. Photo of section

Intraperitoneal dose of labelled orthophosphate of about 0.25 mC.
Survival time 45 min.
Thickness of section 20 μ.
Time of exposure 4 days.
Very little activity can be seen in the brain.
The contours of the hemispheres, the cerebellum and the brain stem are faintly outlined.
The walls of the ventricular system are also faintly outlined.
The strong radioactivity in the choroid plexus of the lateral ventricles corresponds to the macroscopic blood vessels.
The choroid plexus in the third ventricle has large veins in its vicinity.
The frontal activity comes from the sagittal sinus. It is spread out due to the section being folded locally.

Fig. 3. BBB. Basal section showing activity in the hypophysis. Photo of section

Dose of labelled orthophosphate intraperitoneally about 0.15 mC.
Survival time 35 min.
Thickness of section 20 μ.
Time of exposure 4 days.
The strong activity originates from the skull bone.
Weak activity outside the nervous system comes from muscles or from blood.

B. *BLB*

Autoradiographs showing the choroid plexus of the lateral ventricles illustrate the BLB for radioactive orthophosphate (Fig. 2). There is an intense activity which corresponds to the macroscopically visible blood-vessels in the choroid plexus. This is in agreement with the results obtained with another technique for the choroid plexus of the rabbit (Chapter 2, page 26). The results indicate that the P^{32} is already impeded by the walls of the blood-vessels of the choroid plexus. Autoradiography of the choroid plexus with the technique used here has however the

disadvantage that the plexus shrinks when the sections are dried in the freeze-room, the macroscopic blood vessels being undoubtedly the most resistant part. Despite this objection, it does not seem likely that the activity of the plexus would have corresponded to the macroscopic blood-vessels if the activity had been able to pass freely through the capillaries of the plexus (compare Fig. 21, page 73). In this connection, it may be worth mentioning that the macroscopic blood-vessels of the choroid plexus of the rat are much larger than those of the rabbit.

The intense activity corresponding to the macroscopic blood-vessels in the choroid plexus seems to be stronger than the activity for other similar-sized blood-vessels, *e.g.* the large venous sinuses. Despite the fact that the autoradiographs are not quantitative, this can hardly be a contingency since the same circumstance is observed in all the autoradiographs. From determinations of the turnover rates of different organic phosphate compounds in the blood and in the choroid plexus, BORELL & ÖRSTRÖM (1945) arrived at the conclusion that the activity found in the choroid plexus cannot be wholly attributed to its blood-content, but must be partly ascribed to metabolic processes. This conclusion in conjunction with the present author's observations probably indicates that metabolic processes of a specific nature occur in the walls of the blood-vessels of the choroid plexus.

In cases where radioactive orthophosphate had been injected into the cisterna magna (see this chapter, Fig. 4), the autoradiographs indicated that only small amounts of activity were present in the choroid plexus, in spite of the fact that the activity had entered the ventricular system. None of the tissue elements of the plexus could be distinguished in these autoradiographs. This indicates that the epithelium of the plexus acts as a barrier for orthophosphate in the direction CSF → choroid plexus. The influence of the epithelium on the passage of orthophosphate in the opposite direction is not known. These results also agree with those found using a different technique on rabbits (Chapter 3 page 31). It must be pointed out however that the conclusions drawn from the autoradiographs concerning the role of the epithelium of the choroid plexus in a barrier system are not so

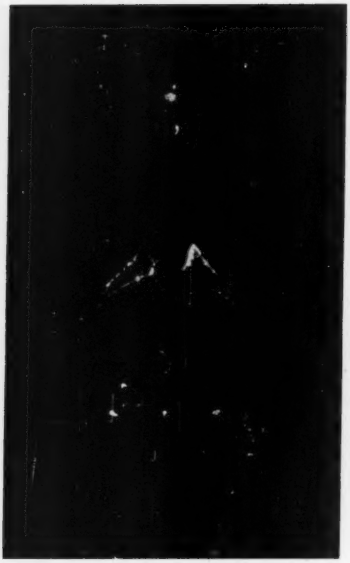


Fig. 4. LBB. Intracisternal dose of P^{32} about 3 μC. Photo of section

Time of survival 60 min.
Thickness of section 20 μ.
Time of exposure 4 days.
The hemispheres, lateral ventricles, third ventricle, Sylvian aqueduct, brain stem and cerebellum are outlined. Entrance into structures remote from the CSF-system cannot be established with certainty.
Very little activity has entered the choroid plexus.

certain as the corresponding conclusions regarding the blood-vessels. The ventricular walls seem to have a strong affinity for orthophosphate, thus making it possible to obtain similar results, assuming that the role of the epithelium is of a passive nature (see this chapter, section C).

C. *LBB*

Autoradiographs obtained after injection of radioactive orthophosphate directly into the cisterna magna clearly illustrate the

Fig. 5. LBB. Same animal as in fig. 4. More basal section. Lower parts of anterior horns connected with Foramen of Monro. Third ventricle, fourth ventricle, brain stem, cerebellum and hemispheres outlined.

LBB after survival times of 1—60 minutes, the results thus corresponding in this case also with those found in the previous chapters for rabbits. The activity seen in the autoradiographs clearly indicates the contours of the brain at the boundaries between the brain and the cerebrospinal fluid. This is also true for the walls of the ventricular system.

Figures 4, 5 and 6 illustrate these facts.

Discussion of the LBB

It is not possible to decide whether any activity can be seen penetrating into the interior of the brain from the bounding surfaces between the brain and CSF since any eventual pene-

Fig. 6. LBB. Intracisternal dose of P^{32} of about 5 μC. Survival time 2 min. Thickness of section 30 μ. Time of exposure 9 days. Cerebellum, brain stem and hemispheres outlined.

tration may be weak and cannot be reliably distinguished from secondary radiation. This indicates that the barrier observed is an effective one during the experimental times (1—60 minutes). On the other hand, the result does not imply that the barrier is an absolute one. On the contrary, it seems probable that the transport of the P^{32} is only delayed in the barrier for reasons that will be given below (this chapter, page 46 and Chapter 6).

It is only in favourable cases that the activity is evenly spread on all the surfaces mentioned. In such favourable cases, the animals were allowed to live a relatively long time after the cisternal injection (30—60 minutes). However, not all the cases with a relatively long survival time exhibit an even distribution. The possibility of getting such even distributions seems to be rather a matter of chance.

In the cases where the animals were killed a relatively short

time after the cisternal injection (1—15 minutes), an even distribution of the activity was never obtained. The activity in these cases was concentrated around the cerebellum and the brain stem and, in addition, some activity can nearly always be seen in the fissura between the olfactory lobes. Apart from the differences mentioned here, all the autoradiographs in this series of experimental animals give the same results, independent of the different times of survival after the cisternal injection.

It is very probable that the activity seen in the autoradiographs, at least in the cases with a relatively long survival time, has for the most part entered into organic compounds in superficial cell-layers. The results of earlier investigators previously referred to (page 28) are in favour of this opinion, as also are the author's results in Chapter 3 since the activity values for the CSF samples, although high compared with those of the other samples, indicate that only a small amount of the injected radioactive orthophosphate still remains in the CSF. Further evidence will be given in Chapters 6, 7 and 8.

A peculiarity common to all the autoradiographs in the present series is that the activity after one single injection always concentrates in typical spots as is evident from the autoradiographs given here. These spots are present even in the cases where only small amounts of the activity had entered the subarachnoidal space. The reason for this is not clear but is probably of a technical nature. Thus, a rather strong emitter of β-rays is injected directly into the subarachnoidal space and cannot escape far from this space since it is impeded in cell-layers forming the bounding surfaces between the CSF and the CNS. Under such conditions it seems reasonable to presume that there will be great variations in the activity collected from small cisterns and, on the other hand, from narrow spaces containing only minute amounts of CSF. In this way an excess of activity may be obtained on autoradiography in some places and a lack of activity in other places. The author has observed lately that the spot formation of the autoradiographic radioactivity in the LBB can be avoided if larger doses of P^{32} (about 8 μC) are used on a film of fine grain (Agfa Printon).

In section B of this chapter, a weak but definite activity was

observed in the walls of the ventricular system, probably due to P^{32} which had passed the BLB. This activity however shows no spot formation of the type dicussed here.

Active transport of P^{32} from the surface to the interior of the brain

As mentioned before (Chapter 3, page 27), SACKS & CULBRETH (1951) and BAKAY (1956) were of the opinion that the brain may get an essential part of its orthophosphate requirements via the BLB. The existence of a barrier towards orthophosphate between the CSF and the CNS supports this opinion since there are reasons for believing that this barrier includes metabolic processes perpetually occuring in cell-layers bordering the cerebrospinal fluid system (see Chapter 3, page 32 and the present chapter, page 45).

It seems probable that such metabolic processes occurring on the boundary surfaces do not definitely prevent the transport of P^{32} to the deep structures of the brain. The barrier observed may only delay the transport of P^{32} in such a way that the orthophosphate rapidly enters into organic compounds in the outer cell-layers. From there, the P^{32} may be transported by further metabolic processes, as well as by diffusion, and may gradually enter the deep structures of the brain.

In order to obtain evidence for this hypothesis, the author produced autoradiographs on the rat's brain in two cases with a survial time of 24 hours following the intracisternal injection of about 0.02 mC radioactive orthophosphate. In these cases, the autoradiographs showed no trace of the barrier seen in the experiments with the relatively short survial time of up to 60 minutes. After 24 hours the activity had penetrated the brain diffusely as can be seen in Fig. 7. For comparison see discussion in Chapter 3, page 32.

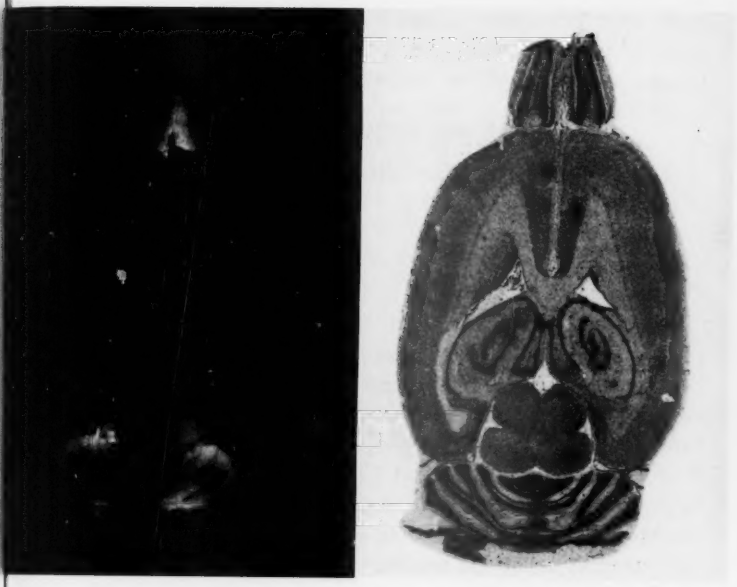


Fig. 7. LBB. Photo of section

After a survival-time of 24 hours the LBB is no longer visible.
P^{32} has entered deeply into the brain.
Intracisternal dose of P^{32} of about 0.02 mC.
Thickness of section 20 μ.
Time of exposure 4 days.

CHAPTER 5

Autoradiographic Studies on the Dissolving of the LBB in Rats

In the two previous chapters the existence of a barrier towards radioactive orthophosphate between the CSF and the CNS was demonstrated in the rabbit and the rat. Reasons were given in support of the opinion that this barrier acts as a metabolic process in cell-layers of the CNS bounding the cerebrospinal fluid system (see Chapter 3, page 32 and Chapter 4, page 45). The present author considered that further evidence in favour of the theory would be obtained if one could get, by destroying the superficial cell-layers of an active barrier, a diffuse penetration of P^{32} into the brain even after a relatively short survival time following the intracisternal injection of radioactive phosphate.

Among substances stated to have a dissolving effect on barriers in the CNS, bee venom attracted the interest of the author. This venom contains enzymes with a lipoid-splitting action and has earlier been used in investigations on metabolic processes occuring in biological boundaries (ÖHMAN 1944). The lipoid-splitting enzymes are effective in very small quantities, thus probably being also able to prevent metabolic processes in the deeper brain structures as they gradually penetrate into the interior together with P^{32}.

It can be thought that more than one of the outer cell-layers of the brain bounding the cerebrospinal-fluid system possess special metabolic functions in the LBB process. Considering that both the walls of the ventricular system and the exterior surfaces of the brain act as barriers towards radioactive phosphate, it is likely that both the ependyma of the ventricular system and the membrana limitans gliae of the exterior surfaces of the brain play an important part in the barrier process between the CSF

and the CNS. Although the histological structures of both kinds of cell show differences, they have certain fundamental properties in common, *e.g.* both are of glial origin and both have developed into covering cell-layers in the bounding surfaces of the CNS. Because of their histological structures, it has earlier been considered that both the membrana limitans gliae (Nageotte 1910) and the ependyma (v. MONAKOW & MOURGUE 1928) may have secretory functions. Ultramicroscopic investigations in this field recently performed by DEMPSEY & WISLOCKI (1955), support the opinions given here.

Experimental

Apisan was the first substance used to dissolve the LBB. Apisan is a Swedish preparation of bee-venom in a concentration of 0.1 %.

About approximately 2—5 μC of radioactive orthophosphate was injected intracisternally into three animals using the same technique as before (page 35), with the difference that 0.01 ml apisan was used instead of physiological saline. Autoradiographs were obtained as before (page 36) after survival times of 5—65 minutes.

As mentioned above, apisan contains *inter alia* several lipoid-splitting enzymes, among them lecithinase. By courtesy of the Nobel Institute for Biochemistry, the author was able to examine the action of β-lecithinase in as pure a form as can be obtained at the present time (crystalline). The β-lecithinase was injected intracisternally in a solution of physiological saline together with 2—5 μC labelled orthophosphate into five animals, the survival time being 50—60 minutes. The volumes injected in each case were approximately 0.02 ml, each containing about 0.04 mg β-lecithinase.

The protein-splitting enzyme trypsin was examined for the same purpose and with the same technique as that for β-lecithinase, the quantity of trypsin was about 0.1 mg in a volume of 0.02 ml. Survival times were 60 minutes (two animals) and 70 minutes (one animal).

Similar experiments were performed on the BBB and the BLB. For this purpose, a solution of physiological saline containing

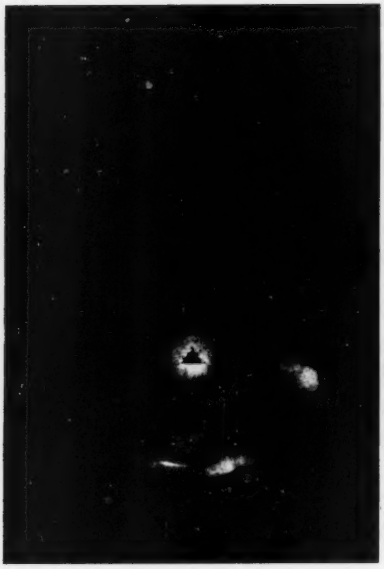


Fig. 8. Dissolving of LBB by β-lecithinase (0.04 mg). Photo of section

P^{32} enters diffusely into the brain.
Dose of P^{32} about 4 μC. Survival-time 60 minutes. Thickness of section 15 μ. Time of exposure 6 days.

radioactive orthophosphate and the enzyme was injected intravenously. The doses of P^{32} were of a similar magnitude as those in Chapter 4, section A—the amounts of the enzymes were, however, about one hundred times greater than the corresponding doses in the intracisternal experiments of this chapter.

Results

A. *LBB*

The autoradiographs in all experiments with apisan, β-lecithinase and trypsin (*e.g.* Fig. 8 and 9) show the same thing, *i.e.* a diffuse penetration of the P^{32} due to a dissolving of the barrier between the CSF and the CNS.

Fig. 9. Dissolving of LBB by trypsin (0.1 mg). P^{32} enters diffusely into the brain. Dose of P^{32} about 4 μC. Survival time 60 min. Thickness of section 15 μ. Time of exposure 4 days.

B. *BBB*

No signs of a dissolving action on the BBB were observed, the autoradiographs exhibiting no obvious differences with the corresponding autoradiographs in Chapter 4, (Fig. 2). Experiments with still larger doses of enzymes or other substances presumed capable of destroying metabolic barriers or with other technical approaches to the problem were not attempted since problems in connection with the BBB are of secondary importance for the present work.

Discussion

It is probable that the minimal doses of lecithinase necessary for a dissolving effect on the LBB are smaller than the doses

actually employed—these minimal doses have not been ascertained since such investigations are not important for determining the nature of the barrier and since β-lecithinase is very expensive and difficult to obtain. Both apisan and β-lecithinase showed toxic effects on the animals. After the intracisternal injection, there was an interval of several minutes before the animals began to show neurological symptoms in the form of muscular twitches and a general spasticity. On the other hand, no toxic symptoms could be seen in the trypsin experiments.

CHAPTER 6

Metabolic Processes in the LBB Elucidated Using Measurements on P^{32} Disappearance from the CSF in the Rabbit

In the previous chapter it was shown that the barrier towards radioactive orthophosphate between the CSF and the CNS can be dissolved by introducing enzymes into the cerebrospinal fluid. The results are in agreement with the theory that the delayed transport of orthophosphate into the CNS implies a metabolic process, since it is reasonable to assume that the enzymes employed disturb the active processes presumed to occur in the covering cell-layers bounding the cerebrospinal fluid system.

On the other hand, one may object that the observations in favour of a metabolic process are only circumstantial evidence. However, the present author considers that the observation of LINDBERG & ERNSTER (1950) that P^{32} had entered into organic compounds in the CNS only two minutes after the injection of radioactive orthophosphate into the subarachnoidal space, combined with the fact that there exists a barrier between the CSF and the CNS, can be regarded as more direct evidence for the existence of a metabolic process in one or several cell-layers covering the CNS and bounding the cerebrospinal fluid system. An attack on the problem from another point of view seemed desirable. The author therefore decided to examine substances with a restraining effect on catalytic activities in intermediary metabolism with regard to their influence on the P^{32} content in the CSF following intracisternal injection.

The role of sulfhydryl groups (-SH groups) in intermediary metabolism

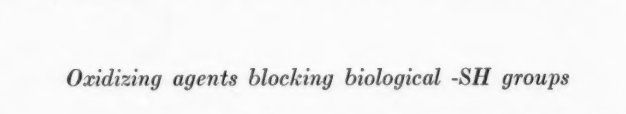
The importance of -SH groups in many biological substances is well-known. For some enzymes, some fraction at least of the substituent -SH groups must be maintained as such, in the reduced form, in order that the catalytic activity shall remain unimpaired. The role of the -SH groups in the catalytic processes is not yet clear. The importance of -SH groups in intermediary metabolism has however been proved in numerous investigations of many different kinds (For reference see BARRON 1951).

Oxidizing agents blocking biological -SH groups

Among agents that block biological -SH groups and are suitable for the experiments in this chapter and Chapters No. 7 and 8, two substances, *viz.* *p*-chloromercuribenzoate sodium and *N*-ethylmaleinimide, have been chosen. The first belongs to a group of mercury derivatives forming mercaptides on reaction with -SH groups according to:

$$\mathrm{R\,SH + R'HgX \rightarrow RS\,Hg\,R' + H^{+} + X^{-}}$$

where X^{-} is an anion (for *p*-chloromercuribenzoate it is chloride) and R, R^1 represent the inactive parts of the molecules in the reaction. The second substance, *N*-ethylmaleinimide, belongs to a group of alkylating agents reacting with -SH groups according to:



This short review concerning -SH groups in biological substances and derivatives blocking the -SH groups has been derived in part from the work of CHINARD & HELLERMAN (1954).

Experimental

Surgical procedure

Rabbits were used—the technical procedure in these experiments is a difficult one and requires strict control if reliable results are to be obtained. Because of this, it was necessary to obtain a smooth narcosis. For this purpose the animals were anaesthetized with urethane intravenously injected as a 20 % solution in a dose of 7 ml per kg body-weight. The membrana atlanto-occipitalis was exposed using the neurosurgical technique, thus securing an absolutely bloodless field.

Intracisternal injections

The technique described in Chapter 3 was employed for all the intracisternal injections, the volume injected amounting to 0.1 ml. In the standard sample of normal cases, only labelled orthophosphate in physiological saline was injected. In the first test series, *p*-chloromercuribenzoate sodium was added to the labelled orthophosphate and, in the second, *N*-ethylmaleinimide. To determine whether the SH-blocking (see results) may be due to a non-specific effect, *p*-chloromercuribenzoate was used in three cases after it had been specifically neutralized by glutathione (BARRON 1951).

Technique for taking CSF-samples

Two minutes after intracisternal injection, a uniform distribution of P^{32} in the CSF was considered to have been established. This time was chosen as time of zero. CSF-samples were taken at regular intervals, *viz.* at 0, 3, 8, 13, 18, 28 and 38 minutes. In some instances, the early samples were collected more often, *viz.* at 0, 1, 2, 3, 4, 5, 8, 13, 18, 28 and 38 minutes. The CSF-samples were obtained using a self-filling micro-pipette of 2,66 μl capacity. Immediately before the collection of a CSF sample, the membrana atlanto-occipitalis was wiped with a piece of cotton wool moistened with physiological saline. A suction tube and a pair of pincers were used during this procedure as in ordinary neurosurgical operations. A small amount of CSF can be seen oozing out from the puncture and is im-

mediately collected in the micro-pipette. During the intervals between the samples, a small piece of beaten muscle is gently pressed against the puncture in order to prevent a leakage of CSF. After wiping the membrana atlanto-occipitalis, it often happens that no CSF oozes out spontaneously. In such cases, CSF can be obtained by repuncturing at the same hole with a clean needle. The samples are at once transferred to aluminium dishes, each containing a small filter-paper which is subsequently used for the measurements of radioactivity.

Technical comments

After removing the syringe, there will be an immediate leakage, varying in degree, through the hole. However, it is desirable to withdraw the syringe immediately after the injection, thus permitting the leakage, in order to prevent the formation of a false depot. The leakage very soon stops by itself. Some of the injected substance always enters the CSF and there diffuses rapidly. The absolute number of counts obtained are different for different experiments. However, this is without importance since the amounts of orthophosphate injected are small and do not influence physiological concentrations. The concentrations of the agents blocking the -SH groups will also be different due to leakage. The concentrations calculated are therefore maixmum values. It is difficult to state the minimum concentrations necessary to obtain significant results. No toxic effects could be observed during the SH-blocking experiments.

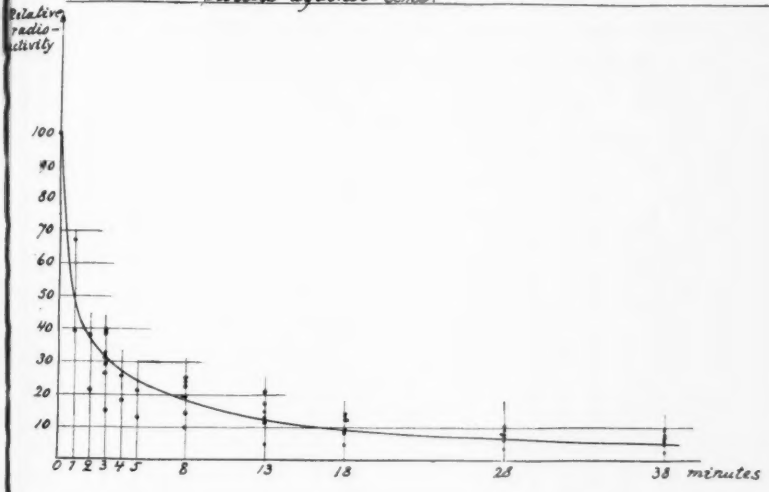
It is not recommended to withdraw a corresponding volume of CSF before the injection of the test substance since experience shows that it is difficult to get CSF into the injecting syringe after a second puncture, thus increasing the risk of getting a false depot.

Results

There is a significant difference between the standard series and the test series where an agent blocking -SH groups was added. In the cases where -SH groups had been blocked, the absorption of P^{32} from the CSF is delayed in the early parts of

Fig. 10

The disappearance of P^{32} from the CSF in seven normal rabbits. Relative radio activities are plotted against time.



the experiments, indicating the restraining effect on the metabolic process in tissue-layers bounding the cerebrospinal fluid system. The detailed results are as follows.

A. *Pure radioactive orthophosphate in the CSF*

In the standard cases (seven in number, Fig. 10), the relative radioactivities decreased rapidly at the beginning but this decrease became gradually less with increasing time. Thus, in the later parts of the experiments, the P^{32} values are decreasing slowly, approaching zero asymptotically.

B. *Radioactive orthophosphate plus agents blocking -SH groups*

In these cases (eight in number, Figs. 11, 12) the SH-inhibitor had a maximum concentration in the CSF of approximately

Fig 11

The disappearance of P^{32} from the CSF in five rabbits under the influence of p-chloromercuribenzoate sodium. Relative radio activities are plotted against time (five cases). Maximum concentration of p-chloromercuribenzoate sodium in CSF = 10^{-3} M.

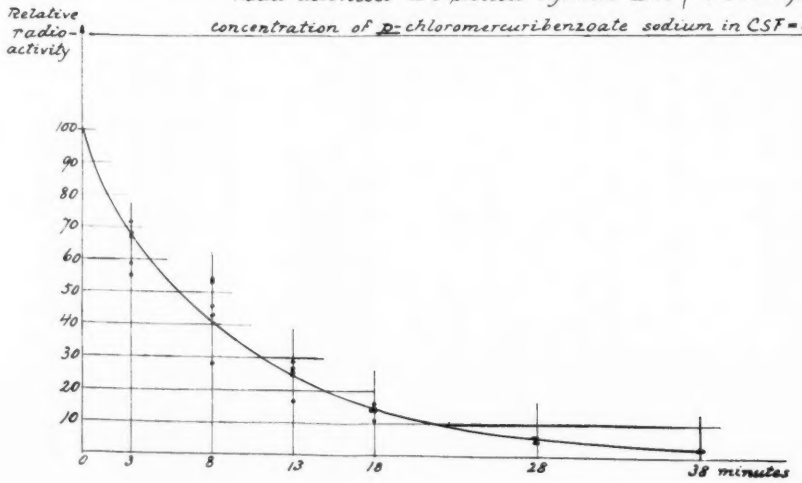


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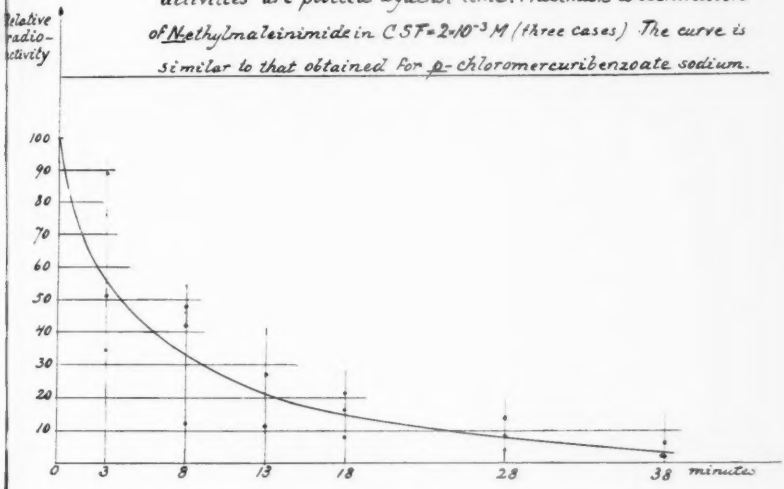
Fig. 12

The disappearance of P^{32} from the CST in three rabbits under the influence of N-ethylmaleinimide. Relative radioactivities are plotted against time. Maximum concentration of N-ethylmaleinimide in CST = 2×10^{-3} M (three cases). The curve is similar to that obtained for p-chloromercuribenzoate sodium.



1—2 $\times$ 10^{-3}M (*p*-chloromercuribenzoate five animals and *N*-ethylmaleinimide three animals). The normal amount of CSF in the rabbit is about 1.5 ml. The disappearance of the P^{32} from the CSF was decreased in the early parts of the experiments. With increasing time, the P^{32} values tend to approach equilibrium faster than is typical for the corresponding P^{32} values in the normal cases. The results are very similar for both the SH-inhibitors examined.

C. *Radioactive orthophosphate plus p-chloromercuribenzoate and glutathione*

To determine whether the SH-blocking may be due to a non-specific effect, *p*-chloromercuribenzoate was used in the same concentration as in the ordinary experiments (10^{-3}M), with the

Fig. 13

The disappearance of P^{32} from the CST in three rabbits under the influence of p-chloromercuribenzoate sodium and in addition glutathione in the proportions 1 M / 1,5 M (three cases). Relative radioactivities are plotted against time. The curve has the same character as that obtained in the normal cases.

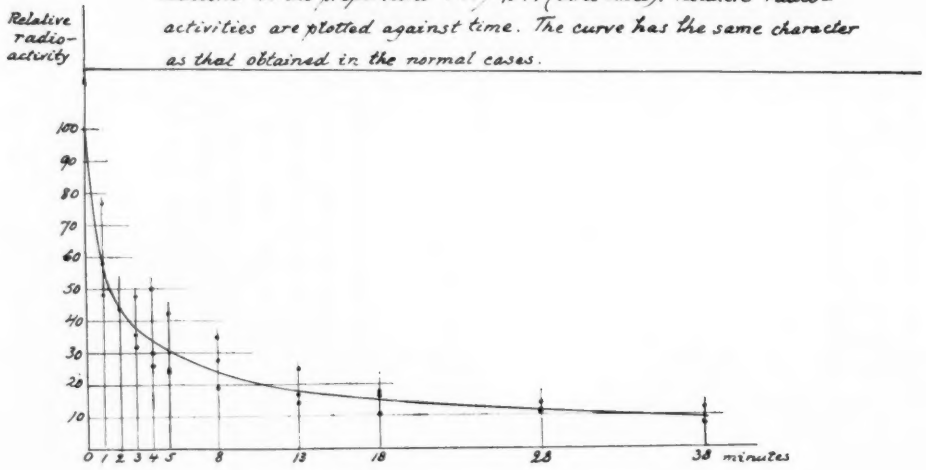


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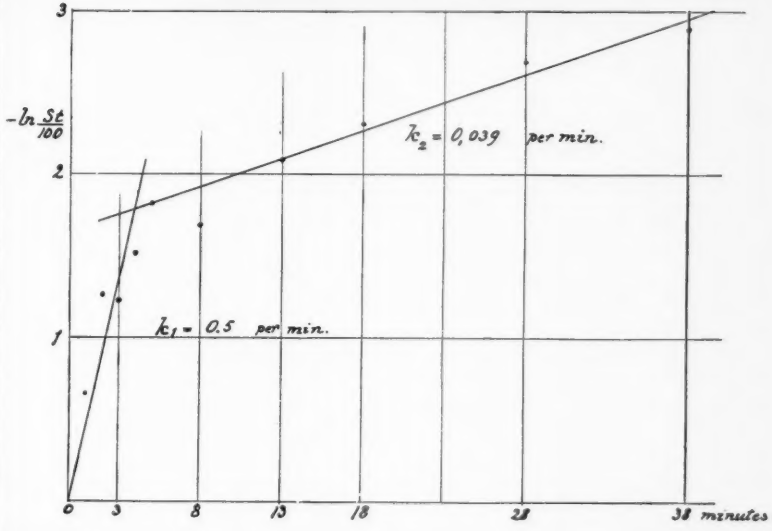
Fig. 14 (derived from Fig. 10)

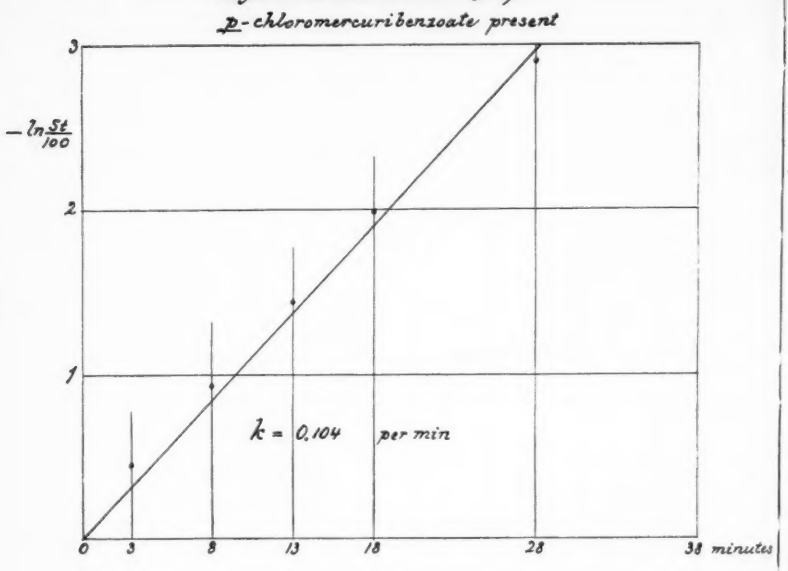


difference that the *p*-chloromercuribenzoate had in its turn been blocked, as regards its inhibitory action, by the addition of an excess of reduced glutathione. The proportions were 1.5 moles of glutathione per mole of *p*-chloromercuribenzoate (three cases, Fig. 13).

The results indicate that the disappearance of P^{32} from the CSF in these cases is of the same character as that for the normal cases where no inhibitor was employed.

Mathematical analysis of results

It appears from the figures 10—13 that the relative radioactivity in the CSF (S_t) decreases asymptotically to zero. This suggests that an exchange of phosphate occurs between the CSF and reservoirs, the phosphate contents of which are much larger than that of the CSF.



The rate of phosphate exchange (k) is then a function of time ($t =$ min.) and relative radioactivity (S_t), according to the equation

$$-\ln\frac{S_t}{100} = k \cdot t,$$

and can be computed from the slope of the line $-\ln\frac{S_t}{100}$ as plotted versus t. These plots are shown in Figs. 14—17, calculated on the basis of average values derived from Figs. 10—13, respectively. Fig. 14 represents the normal series. Figs. 15 and 16 represent the series injected with the SH-inhibitors, *p*-chloromercuribenzoate and *N*-ethylmaleinimide respectively. Fig. 17 represents the series treated with *p*-chloromercuribenzoate + glutathione.

Only when SH-inhibitors were present in an active form (Figs. 15 and 16) were straight lines obtained. The value of k was estimated to be 0.093 per min. in Fig. 15, and 0.104 per min. in

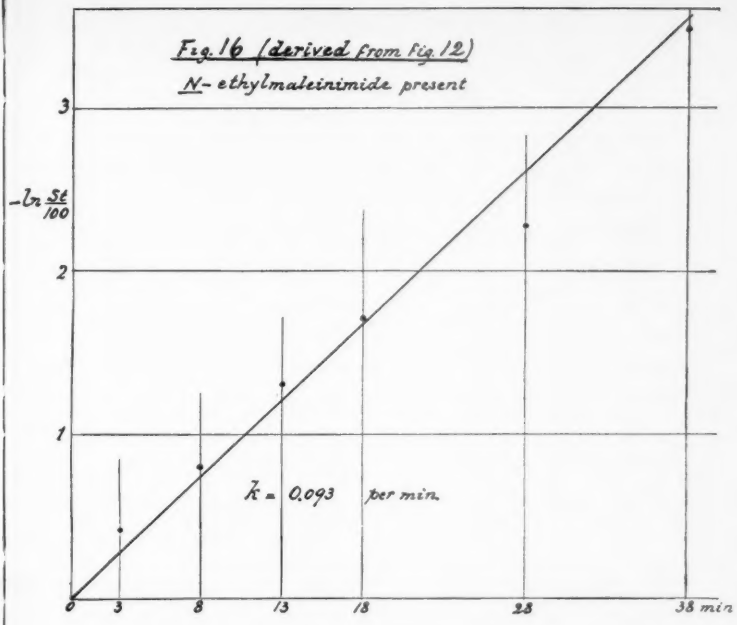
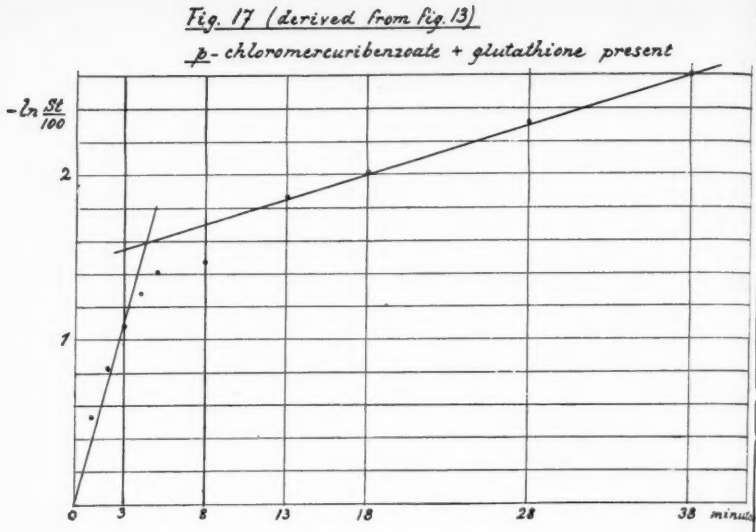


Fig. 16, giving an average value of approximately 0.1 per min. for both SH-inhibitors.

The normal case (Fig. 14) gives a curve which rises first steeply, then breaks off at a certain level and continues as a slowly ascending straight line. Fig. 17 (*p*-chloromercuribenzoate + glutathione) is similar to Fig. 14. The shape of these curves suggests that, in these cases, the radioactive phosphate underwent a rapid exchange and reached equilibrium with a tissue-layer after about 4 minutes. The phosphate content of this tissue-layer is of the same order of magnitude as that of the CSF, as indicated by the level at which the break-off takes place.

The rate of exchange for this process (k_1) can be computed from the initial slope of the curve, yielding a value in the case of Fig. 14 of about 0.5 per min. The rate of exchange with the "large phosphate reservoirs" (k_2) can be estimated from the slope after the break-off to be about 0.04 per minute.



From these calculations, it appears that under physiological conditions there occurs a rapid phosphate exchange between the CSF and cell-layers bounding the CSF. These cell-layers may constitute barriers which delay transport of phosphate. The barrier mechanisms can be broken by SH-inhibitors. That this effect is specific is shown by the fact that it is neutralized by glutathione.

Under physiological conditions, the phosphate content of the CSF is lower than that of the blood. This indicates that the barrier mechanisms require energy to be maintained, which may explain the importance of free -SH groups for their maintenance.

It can be expected that the phosphate content of the CSF increases in the presence of SH-inhibitors. The phosphate content of the CSF was determined in five rabbits. Physiological saline (0.1 ml) was injected into the cisterna magna 30 minutes before the CSF sample was taken. In another series of five rabbits, 0.1 ml of a solution of *p*-chloromercuribenzoate was injected into the cisterna magna, giving a SH-inhibitor concentration of about 10^{-3}M, 30 minutes before the CSF sample was taken.

Table 3

Normal cases			SH-inhibitor cases		
Rabbit		Phosphate content in CSF in mg% P	Rabbit		Phosphate content in CSF in mg% P
No.	Weight kg.		No.	Weight kg.	
1	2.1	0.71	1	1.5	4.49
2	2.1	0.68	2	2.3	4.34
3	2.3	0.93	3	3.1	3.10
4	4.0	0.84	4	3.9	3.72
5	3.4	1.24	5	2.5	2.76

The results are given in Table 3.

The average phosphate content in the normal cases is 0.88 mg % and in the SH-inhibitor cases 3.68 mg %.

The normal values obtained are in agreement with those found in the literature. The values obtained in the SH-inhibitor treated animals are, on the other hand, on the same level as the orthophosphate concentration in rabbit blood plasma (for reference see PALM 1948, page 30).

When the barriers are broken down by SH-inhibitors, the turnover rate of phosphate in the CSF increases, probably largely due to an increased exchange with the blood.

The absolute phosphate exchange rates (v) can be calculated from the values of k, obtained above, as follows:

1. *Normal animals:*

$k_1 = 0.5$ per min.; $v_1 = 0.5 \times \frac{0.88}{100} = 4.4$ μgram P per min. per gram CSF.

$k_2 = 0.04$ per min.; $v_2 = 0.04 \times \frac{0.88}{100} = 0.35$ μgram P per min. per gram CSF.

2. *SH-inhibitor treated animals:*

$k = 0.1$ per min.; $v = 0.1 \times \frac{3.68}{100} = 3.68$ μgram P per min. per gram CSF.

Fig. 18

Normal case

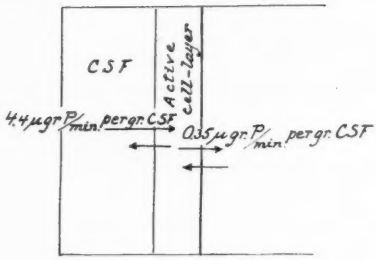
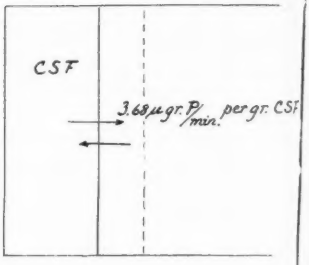


Fig. 19

SH-inhibitors present



A comparison of the different values of v obtained indicates that the rate of phosphate exchange between CSF and the active cell boundaries which act as barriers under physiological conditions is of a similar magnitude to that of the phosphate exchange between the CSF and the large reservoirs in the cases where SH-inhibitors were present. Physiologically, the barriers decrease the phosphate exchange rate from the CSF from 4.4 μgram P per min. per gram CSF to 0.35 μgram P per min. per gram CSF, or, in other words, by a factor of about ten. This mechanism is broken by SH-inhibitors. This is illustrated in Figs. 18 and 19.

The term "large reservoirs" used in the above mathematical analysis includes, obviously, both blood and brain and it has not been possible to distinguish between BLB and LBB. It may appear probable, however, that the active boundaries, the existence of which could be deduced from this analysis, function as barriers for phosphate exchange both between CSF and blood and between CSF and CNS.

These results agree well with those of TSCHIRGI and TAYLOR (1953, 1954). These authors observed a steady potential difference of 5—30 mv between venous blood and the CSF (negative) of the cisterna magna in lightly anesthetized rats, cats and dogs. They suggested that the potential might be due to the activity of some pan-vascular membrane between the blood and the CSF.

CHAPTER 7

Studies on the Total Distribution of P^{32} in the Rabbit Following Intracisternal Injection of Radioactive Orthophosphate

In Chapter 6, conclusive evidence has been presented in support of the view that the barriers towards orthophosphate in the cerebrospinal fluid system involve active transport mechanisms of metabolic nature. This was done by restraining the activity by use of agents blocking biological -SH groups.

Some incomplete observations, not mentioned before and incidental to the experiments on rabbits in Chapter 3, indicated that a relatively small part of the P^{32} after intracisternal injection of radioactive orthophosphate can be found in the body outside the CNS. This probably indicates that the active tissue-layers constituting the barriers of the CSF system absorb most of the radioactive orthophosphate injected. In such a case, it is probable that radioactive orthophosphate in the presence of SH-inhibitors may leave the CSF, being preferentially diverted to the blood. The results in the preceding chapter support this hypothesis. Because of this, it was desirable to ascertain how P^{32} is distributed between the CNS and the rest of the body in normal cases and in SH-inhibited cases.

Experimental

Small rabbits weighing 1.0—1.7 kg were used. The animals were anaesthetized, the membrana atlanto-occipitalis exposed, and a dose of radioactive orthophosphate injected intracisternally employing the technique described in Chapter 6. There was, however, one technical difference: the injection needle was kept

in position for one minute after the injection in order to ensure that sufficient doses were injected into the CSF. For comparison, an equal volume of radioactive orthophosphate solution was injected into a bottle containing non-active phosphate solution. For reasons given in Chapter 6, however, it is not possible to ensure that the doses of radioactive orthophosphate in the CSF and the bottle are exactly the same, the total P^{32} values found in the animals being more reliable.

The animals were allowed to live for one hour after the intracisternal injection and were then killed by air embolism. The skin was stripped off and discarded since it only contains trifling amounts of P^{32} (COHN & GREENBERG 1938). The paws were also rejected.

The CNS was exposed by removing the bone covering the convexity of the skull and the spinal arches covering the medulla spinalis. The CNS was then completely removed. Finally, the animal was decapitated closely cranial to the atlas arch. P^{32} determinations on the CNS, the skull and the rest of the body were then performed separately.

The CNS was homogenized by grinding in physiological saline after which the whole of it was transferred to a bottle containing about 300 ml 10 % trichloracetic acid (TCA). The skull and the rest of the body were then separately put through a meat mincer. The samples thus obtained were transferred to bottles containing 10 % trichloracetic acid, about 0.5 litre for the skull and 3.5 litre for the rest of the body. The bottles were sealed and left for 48 hours. Longer periods of extraction did not yield significantly different values for the total P^{32}, nor did the addition of hydrochloric acid (in order to secure a decalcification of the bony tissues) have any effect. During the extraction, the bottles were shaken vigorously a couple of times each day.

After extraction, 5 ml samples were collected from each bottle and, after filtration, 0.2 ml of each sample was transferred to counting dishes for estimation of P^{32}. This was done three times, an average value being then taken. The total amounts of P^{32} in the different parts of the body were then calculated using the total volumes of substance in the bottles. The results obtained cannot, of course, be expected to agree completely with the real

Table 4

Rabbit		Total P^{32} in million counts per minute and in per cent							Solution of active phosphate, million counts per minute
No.	Weight kg.	CNS	%	Skull	%	Rest of body	%	Total sum	
1	1.1	4.9	55.6	2.3	26.2	1.6	18.2	8.8	6.6
2	1.2	4.5	54.9	2.3	28.0	1.4	17.1	8.2	11.5
3	1.7	5.0	56.2	2.1	23.6	1.8	20.2	8.9	9.6

values since this would imply a uniform composition in the extraction bottles, a condition which is not possible to fulfil. Since, however, the technique employed is the same in all cases, the results obtained can be compared in principle and this is all that is required in this work. For comparison, samples were sometimes taken at different times from the extraction bottles. The results thus obtained were always of the same magnitude.

Three series of investigation were performed. In the first, radioactive orthophosphate in a volume of 0.1 ml physiological saline was injected intracisternally. In the second and third, the influence of *p*-chloromercuribenzoate (10^{-3}M) and apisan (0.1 ml) was determined.

Results

The results are given in tables 4, 5 and 6.

Table 5

Rabbit		Total P^{32} in million counts per minute and in per cent							Solution of active phosphate, million counts per minute
No.	Weight kg.	CNS	%	Skull	%	Rest of body	%	Total sum	
1	1.6	5.8	16.0	8.6	23.8	21.8	60.2	36.2	42.3
2	1.1	3.3	13.2	5.2	20.8	16.5	66.0	25.0	37.4
3	1.0	3.1	23.1	2.3	17.2	8.0	59.7	13.4	9.4

Table 6

Rabbit		Total P^{32} in million counts per minute and in per cent							Solution of active phosphate, million counts per minute
No.	Weight kg.	CNS	%	Skull	%	Rest of body	%	Total sum	
1	1.3	13.9	37.7	9.0	24.4	14.0	37.9	36.9	38.0
2	1.6	14.4	39.3	9.5	25.8	12.8	34.9	36.7	36.7

A. *Pure radioactive orthophosphate (Table 4)*

Of a total of 8.6 million counts, 55.6 % were found in the CNS, 25.9 % in the skull and 18.5 % in the rest of the body. (Note: here and in the following, average values are given.)

B. *Radioactive orthophosphate plus p-chloromercuribenzoate (Table 5)*

Of a total of 24.9 million counts, 17.4 % were found in the CNS, 20.6 % in the skull and 62.0 % in the rest of the body.

C. *Radioactive orthophosphate plus apisan (Table 6)*

Of a total of 36.8 million counts, 38.5 % were found in the CNS, 25.1 % in the skull and 36.4 % in the rest of the body.

Discussion

The distribution differences in Tables 4 and 5 confirm the conclusions above (Chapter 6) concerning the role of the SH-blocking agent in restraining the metabolic processes. The rapid disappearance of the P^{32} from the CSF in the later parts of the curves (Figs. 11 and 12) under the influence of agents blocking the -SH groups is explained by the fact that most of the P^{32} leaves the CSF to be found again in the rest of the body.

CHAPTER 8

Autoradiographic Studies of the LBB in the Rat under the Influence of Agents Blocking Biological —SH Groups

As in the above, it was desirable to complement the results obtained with SH-inhibitors by means of autoradiograms.

Experimental

Rats were injected intracisternally with radioactive orthophosphate together with either *p*-chloromercuribenzoate sodium or *N*-ethylmaleinimide in order to restrain the metabolic activity in the LBB. The amount of SH-inhibitor injected was calculated to give approximately the same concentration in the CSF as that in the experiments with rabbits (10^{-3}M). The doses of radioactive orthophosphate injected were approximately 6 μC in a volume of 0.02 ml (3 μC in a volume of 0.01 ml proved to be insufficient).

Results

Suitable autoradiographs were obtained in four animals, two for each of *p*-chloromercuribenzoate and *N*-ethylmaleinimide.

The autoradiograms show that the P^{32} penetrates diffusely into the CNS as illustrated in Figs. 20, 21. In addition, traces of activity can be seen in surfaces bounding the CSF. A similar effect is noticed for the subarachnoidal space and the ventricular system (Fig. 21). Only in one of the cases, however, had a considerable amount of P^{32} entered the ventricular system. In this

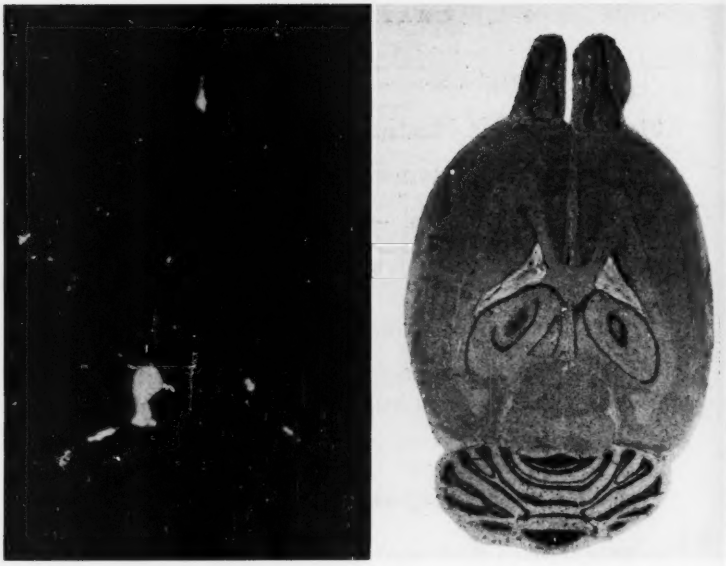


Photo of section

Fig. 20. LBB in the presence of *N*-ethylmaleinimide. Intracisternal dose of P^{32} about 6 μC. Time of survival 60 min. Thickness of section 20 μ. Time of exposure 4 days. Dose of P^{32} greater than ordinary dose for demonstrating the LBB. A diffusion into the brain takes part. Only traces of P^{32} visible in the lateral ventricles.

case, P^{32} had to a great extent entered diffusely into the choroid plexus, a fact possibly indicating that the role of the epithelium of the choroid plexus in the BLB also includes a mechanism of active transport involving metabolic processes (see Chapters 3 and 4).

Discussion

The results agree well with those obtained in the previous chapter since the signs indicating a normal metabolic activity are almost completely absent and since a diffusion of P^{23} into

Fig. 21. LBB in the presence of *p*-chloromercuribenzoate. Dose of P^{32} at least 6 μC. Time of survival 30 minutes. Thickness of section 20 μ. Time of exposure 4 days. Signs of LBB almost eliminated. A diffusion into the brain takes part. P^{32} has entered the choroid plexus diffusely and does not show the macroscopic vessels as in the BLB (Fig. 2).

deeper parts of the brain can be seen. This is what can be expected if the normal metabolism is restrained.

In order to get suitable autoradiographs, however, the author had to prepare several animals owing to the fact that little or no activity could be seen to have entered the subarachnoidal space when small doses 2—3 μC were used. However, no similar difficulties were noticed (see Chapter 5) when apisan and lecithinase were used; P^{32} seemed to diffuse quite easily into the CNS.

This is in conformity with the observation in Chapter 7 that most of the P^{32} leaves the CSF in preference for the blood when SH-inhibitors are present.

The most probable explanation of the differences in the extent of the diffusion into the CNS under the influence of SH-inhibitors

and, on the other hand, apisan and lecithinase seems to the author to be that apisan and lecithinase are active in very small quantities and are thus also able to destroy metabolic functions in the interior of the brain during their diffusion into the interior together with P^{32}. This explanation also seems likely when it is considered that the rats and the rabbits showed toxic symptoms (spasticity and muscular twitches) under the influence of apisan and lecithinase, whereas, on the other hand, similar toxic symptoms due to SH-inhibitors were not evident.

Summary

1. This investigation is concerned with the elucidation of problems of barriers and metabolic processes in the central nervous system (CNS) by means of carrier-free radioactive orthophosphate.

General problems concerning barriers in the CNS are reviewed with special attention to the physiological functions of the barriers. Definitions of the terms "barrier" and "active transport" as used in this work are given. Short reviews concerning the blood-brain barrier (BBB) and the blood-cerebrospinal-fluid barrier (BLB) are given. The site of the BLB is not completely known but it is certain that the choroid plexus plays an important part in it. The selective properties of the barriers and the composition of the CSF indicate that translocation processes are involved during the formation of the CSF which are more complex than just simple barrier processes that are merely able to offer a selective resistance to diffusion. In other words, it seems probable that processes of active transport constitute an important part of the barrier function. It is typical for processes of active transport that they must consume energy in order to be maintained. Hence, metabolic processes in the barriers must be taken into account.

Among ions which have lower concentrations in the CSF than in the blood, PO_4^{3-} appears important because of its participation in many metabolic procesess. It can be presumed that the concentration of PO_4^{3-} in the CSF is related to the presence of metabolic processes in the barriers of the CNS.

2. Using determinations of the specific activities of radioactive orthophosphate in the blood, the choroid plexus, the CSF and the cerebral cortex in living rabbits following intraperitoneal injection of radioactive orthophosphate 1—2 hours earlier, the existence of the BLB and the BBB for orthophosphate was proved. The presence of these barriers towards orthophosphate is previously known. From the present author's results, however, it

appears that a barrier in the BLB system is located in the blood-vessels of the choroid plexus, since the specific activity of the choroid plexus is considerably lower than that of the blood at the same time. The metabolic nature of the barrier in the blood vessels of the choroid plexus is made probable by comparison with other investigators' results on biochemical P^{32}-fractions in the choroid plexus.

3. Specific activities were determined simultaneously for the CSF, the cortex of the CNS and the choroid plexus in rabbits 40—80 minutes after the injection of radioactive orthophosphate in the cisterna magna. The values for the choroid plexus and the CNS were very low compared with that for the CSF.

The conclusion is drawn that orthophosphate meets a barrier during its transport from the CSF to both the choroid plexus and the CNS.

As regards the choroid plexus, the results indicate that the epithelium of the plexus forms a barrier to the penetration of orthophosphate from the CSF to the choroid plexus. Thus, together with the vessels of the choroid plexus, the epithelium also plays a part in the BLB. The nature of this epithelial process is as yet obscure (see page 31, 41 and 79).

The low specific activities obtained in this work for the brain are very significant and indicate the presence of a barrier between the CSF and the CNS (LBB). Earlier investigators have found that radioactive orthophosphate, after injection in the subarachnoidal space, is rapidly transformed into organic compounds in the CNS. The most natural explanation of this transformation, in view of the fact that there is a barrier towards orthophosphate between the CSF and the CNS, seems to be that there exist intense metabolic processes in the cell-layers bounding the cerebrospinal fluid system.

4. The existence of the BLB, the BBB and the LBB in rats was demonstrated autoradiographically by intraperitoneal and intracisternal injection of radioactive orthophosphate, with varying survival times of up to 60 minutes. These studies on a different species of animal using a different technique yielded results comparable with those obtained with rabbits.

As is seen from the autoradiographs, orthophosphate is not

permanently prevented by the LBB from penetrating into the CNS. After a survival time of 24 hours, P^{32} is seen to have entered deeply into the brain of the rat and no trace of the LBB can be detected. The autoradiographic results concerning the LBB are consistent with the hypothesis of a metabolic process in the LBB.

5. Autoradiographs of the brains of rats were obtained using the same technique as above but with the addition of substances containing lipoid-splitting or protein-splitting enzymes to the solutions injected.

Bee venom (apisan) in an intracisternal dose of 0.01 mg completely dissolves the LBB, and the P^{32} penetrates deeply into the CNS even after short survival times of a few minutes. Since bee venom contains *inter alia* lecithinase, the dissolving effect of β-lecithinase on the LBB was investigated. The results obtained corresponded to those obtained with apisan. Trypsin had a similar effect on the LBB.

However, no dissolving effect on the BBB could be observed in autoradiographs taken after the intravenous injection of the enzymes in doses a hundred times larger than the doses which dissolved the LBB.

6. Because of the importance of free -SH groups for catalytic activity in intermediary metabolism, an investigation was performed to determine what influence agents with a blocking effect on biological -SH groups had on the rate at which radioactive orthophosphate dissappeared from the CSF. Two substances having such an effect were studied, *viz.* *p*-chloromercuribenzoate and *N*-ethylmaleinimide. The concentrations of the both substances in the CSF were about $1—2\times10^{-3}$ M.

In the standard series of rabbits, only radioactive orthophosphate was injected intracisternally, after which microsamples of CSF were collected at close intervals over a period of 40 minutes. The P^{32} content was found to decrease rapidly in the beginning of the test period. In the test series, radioactive orthophosphate was injected intracisternally together with either *p*-chloromercuribenzoate or *N*-ethylmaleinimide. In both series, it was found that the early diminution of P^{32} in the CSF was distinctly retarded.

A mathematical analysis of the time curves for the disappearance of P^{32} from the CSF in the different series of animals gave the following results:

Under physiological conditions, there will occur a rapid phosphate exchange between the CSF and bounding tissue-layers. These tissue-layers form barriers which delay the further transport of the phosphate. The barrier mechanisms can however be broken using SH-inhibitors and it is then found that the concentration of orthophosphate in the CSF increases and reaches a value of the same order of magnitude as the normal orthophosphate concentration in the blood of the rabbit. Experimental values for the concentrations of orthophosphate in the CSF in normal cases and in the presence of SH-inhibitors are given. Under the influence of SH-inhibitors, the total phosphate exchange from the CSF is increased, probably mainly to the blood. Under physiological conditions, the phosphate content of the CSF is lower than that of the blood. This indicates that the barrier mechanisms require energy to be maintained. The importance of free SH-groups for the maintenance of these mechanisms is therefore explicable.

The influence of SH-inhibitors is a specific process. This is proved by the fact that *p*-chloromercuribenzoate had no effect when it had been specifically neutralized by means of glutathione.

7. An investigation was performed on the total distribution of P^{32} in the rabbit one hour after intracisternal injection of radioactive orthophosphate. In the standard series, only radioactive orthophosphate was injected. In a test series, radioactive orthophosphate was injected together with an SH-inhibitor (*p*-chloromercuribenzoate) in the same concentration as in the other SH-inhibitor experiments. In the experiments with only radioactive orthophosphate (three in number), it was found that, of an average total of 8.6 million counts, 55.6 % were in the CNS, 25.9 % in the skull and 18.5 % in the rest of the body. On the other hand, in the experiments with radioactive orthophosphate plus SH-inhibitor (three in number), it was found that, of an average total of 24.9 million counts, 17.4 % were in the CNS, 20.6 % in the skull and 62.0 % in the rest of the body.

Two experiments were performed with P^{32} in the presence of

apisan. In these, approximately equal amounts of P^{32} were found in the CNS and the rest of the body. This is in agreement with the autoradiographic results on rat brains which indicated that P^{32} easily penetrates into the CNS in the presence of apisan.

8. The influence of SH-inhibitors (*p*-chloromercuribenzoate and *N*-ethylmaleinimide) on the LBB in rat brains was examined autoradiographically.

Very little sign of the LBB could be seen on the autoradiographs, and the P^{32} was seen to have diffused into the brain. This diffusion process, however, does not take place as easily as the corresponding process under the influence of apisan and lecithinase. This agrees well with the experiments on the total distribution of P^{32} in rabbits (see point 7 in this summary).

In autoradiographs of rat brains in the presence of an SH-inhibitor, it was seen that P^{32} had entered diffusely into the choroid plexus indicating a metabolic barrier function in the epithelium cells of the choroid plexus (compare page 31, 41 and 76).

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